MANIPULATION OF THE LOMA SALMONAE-RAINBOW TROUT DISEASE MODEL WITH SPECIAL REFERENCE TO INTEGRATED MULTITROPHIC AQUACULTURE PRACTICES

A Thesis
Submitted to the Graduate Faculty
in Partial Fulfilment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

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October, 2015

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ABSTRACT

Integrated multitrophic aquaculture (IMTA) can potentially mitigate concerns related to traditional monoculture (*e.g.*, nutrient loading). An ecological approach to nutrient management can diversify the industry while reducing environmental impacts. However, integrated culture systems can potentially harbour pathogens that thrive in complex environments. This thesis explored how an environmentally resistant microsporidial pathogen, *Loma salmonae,* can be used as a model in determining how stable pathogens may behave within an IMTA setting. The work presented in this thesis covers three main themes - all utilizing manipulations of the *L. salmonae*-rainbow trout (*Oncorhynchus mykiss*; LS-RT) disease model.

First, blue mussels (*Mytilus edulis*) were added to the LS-RT model to determine how the added complexity influences disease transmission. When exposed to a suspension of *L. salmonae* spores during a period of feeding, mussels efficiently extracted spores from the water column. Spores remained viable in mussels, retaining infective potential to salmonids for up to 28 days. Mussels also excreted infectious spores in feces and pseudofeces for 24 hours PE. Spores likely become trapped within mantle and gill tissue, accounting for long-term infectivity of exposed mussels. Therefore, blue mussels have the potential to act as a reservoir for *L. salmonae*.

Next, *in vitro* spore viability was correlated to *in vivo* viability using a *L. salmonae* spore specific monoclonal antibody-propidium iodide (MAb-PI) dye exclusion assay. Data revealed correlation between *in vitro* viability and intensity of infection. Several immunocytochemical applications of the MAb protocol were explored, and all showed utility in determining the presence and viability of *L. salmonae* spores in various applications. However, limitations exist when differentiating between viable and empty (*i.e.,* germinated) spores using the MAb-PI protocol.

Lastly, cell culture was integrated into the traditional LS-RT disease model. *L. salmonae* develop xenomas within the rainbow trout gill epithelial (RTgill-W1) cell line. No development was observed in Chinook salmon embryo (CHSE) or mosquito (*Aedes albopictus*; AED) cells. However, AED cells rapidly phagocytize spores while retaining viability and infectivity in rainbow trout (*Oncorhynchus mykiss*). Preliminary studies revealed AED-phagocytozed spores retain *in vivo* infectivity after thawing.

ACKNOWLEDGEMENTS

This is going to be long, as I have had a tremendous amount of support during a non-traditional path through my PhD and veterinary school. I would not have been able to do it without the support and inspiration from many people.

In terms of funding, I greatly appreciate the support this thesis received from the Natural Sciences and Engineering Research Council of Canada (NSERC) through the Canadian Integrated Multi-trophic Aquaculture Network (CIMTAN) in collaboration with its partner Fisheries and Oceans Canada, the University of New Brunswick, Cooke Aquaculture Inc., Kyoquot SEAfoods Ltd, and Marine Harvest Canada Ltd. On a more personal note, the CIMTAN has provided me with a unique funding opportunity I would not have had with any other program. With help from the network I was able to investigate a wide array of scientific problems - from applied to basic, with many tangents in between. I have learned skills that are widely applicable within the aquaculture field, and was also given the freedom to develop my own scientific identity.

I would also like to thank the Atlantic Veterinary College and the 2011 selection committee for granting me the generous AVC Graduate Scholarship. I am honoured to be a recipient. In appreciation, it has motivated me to be the best graduate student I could possibly be. I am indebted to the AVC.

Next I would like to thank my supervisor, Dr. David Speare, for being supportive of my quest to constantly explore the next question. You put a lot of trust in my work and allowed me to investigate many avenues that didn't always clearly fit into the main scheme. This freedom allowed me to feel as though my PhD was my own, worthwhile endeavour. I also appreciate your support in the form of conference travel and teaching opportunities. I will never forget your personal support during a housing issue that without your help, would have probably made me abandon the idea of living on PEI for 7+ years in my very first months here. THANK YOU.

Many thanks to Dr. Glenda Wright for her consistent support in editing and help with learning how to describe stuff properly (perfect example right here). You are a great teacher and I really appreciate your directness and intolerance to unjustifiable helplessness. Thanks to the rest of my committee for their participation and support of my non-traditional path through my PhD: Drs., Fred Kibenge, Sophie St. Hilaire, Rick

Cawthorn and Jeff Davidson. I would not have been able to do it without you all. Along the same lines I must thank Rosemary McIver, Darlene Wakelin, and Dr. Jeffery Wichtel for their support and making the administrative side of things pleasant to deal with.

Thank you to Dr. Dorota Wadowska for her continued support in the TEM lab. Same to Dr. Nicole Guselle and her help in 309N and the aquatics facility - along with the aquatic support staff. Thank you Judy Sheppard for enabling many of my ideas, and also for providing guidance and patience to help focus my excitability in the lab. I must also acknowledge my previous mentors, Drs. J. Randy Jackson, Paul Bowser and Steven Cooke for being integral to my pathway from fisheries management to fish health and veterinary science.

This thesis would have never been written if it weren't for the folks over at UPEI Student Affairs, specifically the Webster Centre for Teaching and Learning. Your services have changed my life for the better. I want to thank you (especially Cathy, Karen, and Marie) for being so accessible, supportive, and helpful in finding resources that allowed me to organize myself enough to facilitate thesis writing.

Of course I must thank my 2 cats, Phoebe and Stanley, who have sat by my side throughout this entire process, often wondering why I haven't fed them on time, but never complaining too much. They were my first companions on PEI and I can't wait to take these island-kitties on whatever adventure is next. Also to Sean, who has complained about not being fed on time, but still loves me regardless. I cannot thank him enough for his support throughout my PhD and veterinary school adventure. I wouldn't have been able to do it without him cheering me on and convincing me that I am capable. All 3 of my furry companions provide constant entertainment and are integral to my personal well-being and subsequent success as a student.

I would also like to thank my family and friends who have all shaped my life in different ways. Especially to my sister Grace who I admire greatly. Even if they are all spread out, I feel very lucky to have such wonderful people in my life.

Lastly I would like to thank my parents, Dan and Martha, for supporting me through all my crazy adventures and undertakings without ever questioning my abilities or choices. You are both an inspiration to me and I love you both very much.

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LIST OF ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BC	British Columbia
CI	Confidence interval
CIMTAN	Canadian integrated multitrophic-aquaculture network
DEX	Dexamethasone
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
FITC	Fluorescein isothiocyanate
GI	Gastrointestinal
HIV	Human Immunodeficiency Virus
IAA	Integrated agriculture aquaculture
IMTA	Integrated multi-trophic aquaculture network
IP	Intraperitoneal
IPNV	Infectious pancreatic necrosis virus
ISAV	Infectious salmon anemia virus
LS-RT	*Loma salmonae*-Rainbow trout
MAb	Monoclonal antibody
MGDS	Microsporidial gill disease of salmonids
OA	*Oncorhynchus* variant
PCR	Polymerase chain reaction
PE	Post-exposure
PoI	Post infection
PI	Propidium iodide
PO	Propylene oxide
PPM	Parts per million
PPT	Parts per thousand
PV	Parasitophorous vacuole
SV	*Salvelinus* variant
TEM	Transmission electron microscopy
VHSV	Viral hemorrhagic septicemia virus
USA	United States of America

1.0 GENERAL INTRODUCTION

1.1 Diversifying Aquaculture

Intensive monoculture is an extremely lucrative form of aquaculture. While productive, many issues exist that are proving difficult to solve. For example, monoculture causes organic and inorganic nutrient loading (Wildish et al., 2004), and associated floral and faunal community changes (Vezzulli et al., 2002; Borja et al., 2009). Due to environmental and microbial community changes, fish density, and subsequent stressors, fishes within monoculture are also particularly prone to disease proliferation (Murray and Peeler, 2005; Borja et al., 2009). These factors, and others, often lead to environmental degradation of farm sites and negative perceptions from potential consumers. There are ways to mitigate these issues by investigating and implementing alternate forms of aquaculture. One promising possibility is the addition of species that utilize wastes from intensively cultured species. Ideally, the added species can allow for increased profits through diversification along with effective biomitigation (Soto, 2009).

1.2 Polyculture

One of the most promising forms of “diversified” aquaculture is the concept of polyculture, where species added to the system feed on waste products from other fed components. Many countries have been utilizing different forms of polyculture for thousands of years. One major example of this is integrated-agriculture-aquaculture (IAA) systems that combine land- and aquatic-based culture. Rice-fish production is common in some parts of Asia, and the addition of fish to rice fields is valuable for pest management (Little and Edwards, 1997), and appears to help maintain natural

biodiversity (Little and Muir, 2003). Livestock-based systems have historically proved to be invaluable in maintaining productivity of small water bodies for tilapias and carps through the addition of livestock waste (Little and Muir, 2003). Similarly, human waste-based systems are common in many urban and rural regions of Asia that lack conventional wastewater treatment facilities (Ghosh, 1990; Pham and Vo, 1990).

Rather than using land-based wastes to increase productivity for cultured fish, aquaponics uses similar concepts, but utilizes aquatic animal wastes to provide nutrients to growing plants. Particulate fish-feed, solid and liquid wastes can provide essential nitrogenous compounds and mineral nutrients essential for plant growth (Wilson et al., 2006). Extracting wastes helps control nutrient loading in recirculating systems and can be conducted at small and commercial scales.

Polyculture is commonly used solely within aquatic systems. Azim and Wahab (2005) define aquatic polyculture as " a management system in which two or more species with complementary feeding habits…are stocked together in the same pond to maximize available food and water resources, thereby obtaining high fish production per unit area of a water body". The polyculture of carps has been widely used for many years in India, Europe, China and other countries (Azim and Wahab, 2005). The basic concept is to stock a carp species that consumes phytoplankton or fed plant material (See Figure 1.1). To avoid the accumulation of organic material, other species are added to consume materials within sediment and substrate (Azim and Wahab, 2005).

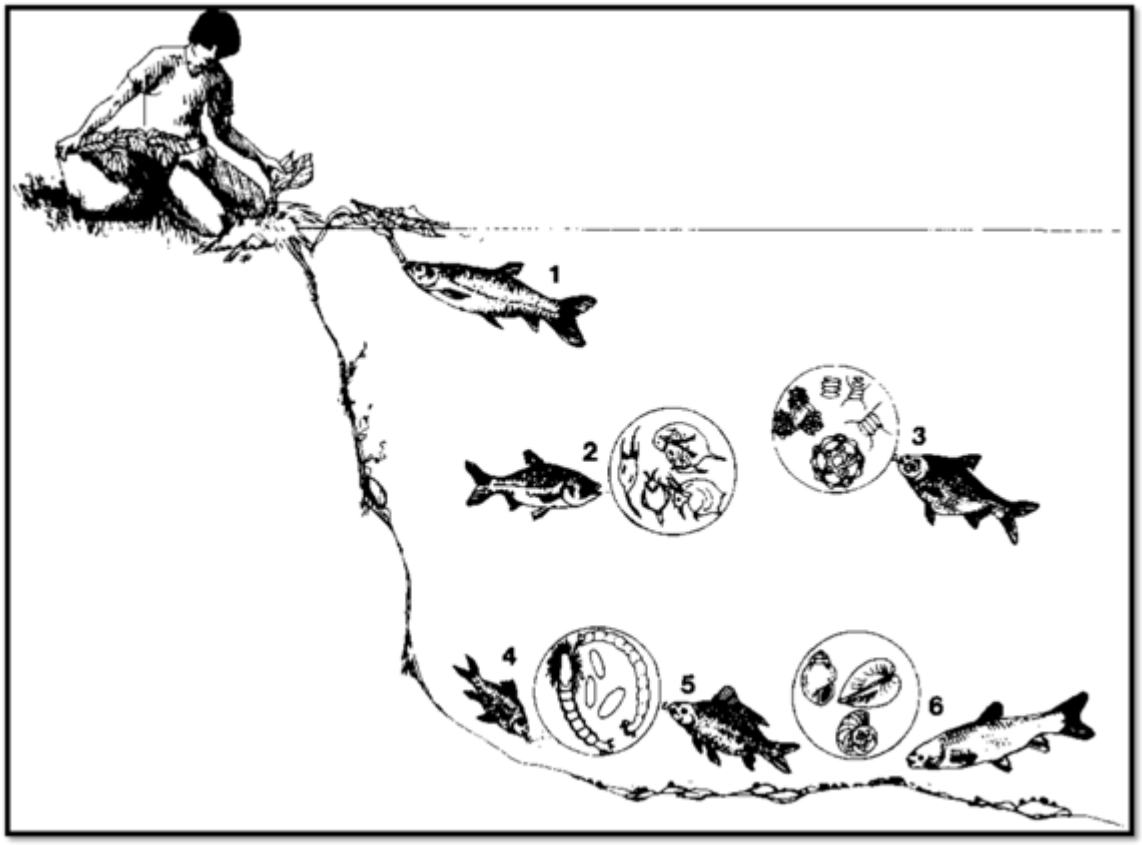

Figure 1.1: Food choices of Chinese carps that occupy different trophic niches: (1) grass carp (*Stenopharyngodon idellus*) being hand-fed vegetable leaves; (2) bighead carp (*Anistichtys nobilis*) eating zooplankton that thrive in productive environments; (3) silver carp (*Hypophthalmichtys molitrix*) eating phytoplankton; (4) mud carp (*Cirrhinus molitorella*) eating benthic animals, detritus and fish feces; (5) common carp (*Cyprinus carpio*) on diet similar to that of mud carp; (6) black carp (*Mylopharyngdon picous*) eating molluscs and benthic invertebrates (From: Bardach et al., 1972).

1.3 Integrated Multitrophic Aquaculture (IMTA)

Polyculture can also refer to any combination of species being cultured in one system. Marketers can take advantage of the term polyculture to give the illusion of a sustainable practice, when in fact they are culturing a combination of species that utilize the same trophic position. In order to combat this potential issue, the term "Integrated Multitrophic Aquaculture" (IMTA) was implemented. IMTA is a form of ecological engineering that aims to increase the economic value and social acceptability of aquaculture while maintaining balanced systems with environmental sustainability (Troell et al., 2009; Chopin et al., 2001). In typical monoculture, uneaten food particles and semi-solid feces fall as organic waste directly below open water net pens (Hargrave, 1995). Inorganic nutrient wastes, such as nitrogen and phosphorus also dissolve into the water column surrounding net pens (Chopin et al., 2001). Both types of wastes alter the community structures in regions surrounding the net pens. In IMTA, fed fin-fish (or shrimps) will likely be the main economic commodity. In theory, particulate waste products from fed species are captured by strategically placed suspension (*i.e.,* filtering bivalves), and deposit (*e.g.,* sea urchins) feeders. Added seaweeds and aquatic plants will utilize inorganic dissolved nutrients within the water column to support their growth (Chopin, 2014). The diversified trophic components should limit ecosystem shifts caused by nutrient loading by one monocultured species (Soto, 2009). Biological and chemical balance can be achieved by utilizing careful proportions of species that provide different ecosystem functions. The major goals are to increase sustainability and ecosystem health surrounding net pens, and increase economic stability and social acceptability (Chopin,

2014). There is a wide range of possible IMTA systems, with many potential species combinations based on local conditions.

1.3.1 The Canadian Integrated Multitrophic Aquaculture Network (CIMTAN)

Salmon monoculture is especially profitable in Canada - which produced 108,118 tons of Atlantic salmon (*Salmo salar*) worth $599,437,000 USD in 2012 (FAO, 2012). In order to diversify farmer's profits and increase sustainability of the farm sites, the feasibility of implementing IMTA sites has been investigated since 2001 (Chopin et al., 2001). In 2010, a Natural Sciences and Engineering Research Council of Canada (NSERC) strategic network was formed uniting university and government scientists with industry partners (Chopin et al., 2013). The Canadian Integrated Multitrophic Aquaculture Network (CIMTAN) was implemented to explore how IMTA could be designed as functioning and profitable farming ventures in Canada. Many aspects of the network employ an interdisciplinary approach in research, development and personnel training in 4 major areas: (1) Ecological design, ecosystem interaction, and biomitigative efficiency; (2) system innovation and engineering; (3) economic viability and societal acceptance; and (4) regulatory science, to facilitate the commercialization of IMTA in Canada (Chopin et al., 2013). Through the network it has been determined that blue mussels (*Mytilus edulis*) efficiently absorb particulate wastes from farmed fish and show high growth rates (Reid et al., 2010; MacDonald et al., 2011). While effective, several other species are being considered to ensure particulate matter that falls to the sediment is captured (*e.g.*, sea cucumber, *Cucumaria frondosa*; green sea urchin, *Strongylocentrotus droebachiensis*; and the basket cockle, *Clinocardium nuttalii*). Two seaweed species,

Palmaria palmata (dulse) and *Ulva* spp. (sea lettuce) are being touted as species that have optimal growth during high inorganic nutrient loading during the Atlantic salmon production cycle (Chopin, 2014). Given the dominance of Atlantic salmon farming in Canada, it is feasible that the addition of filtering bivalves and seaweeds could be successful additions for IMTA. (See Figure 1.2 for a graphic overview).

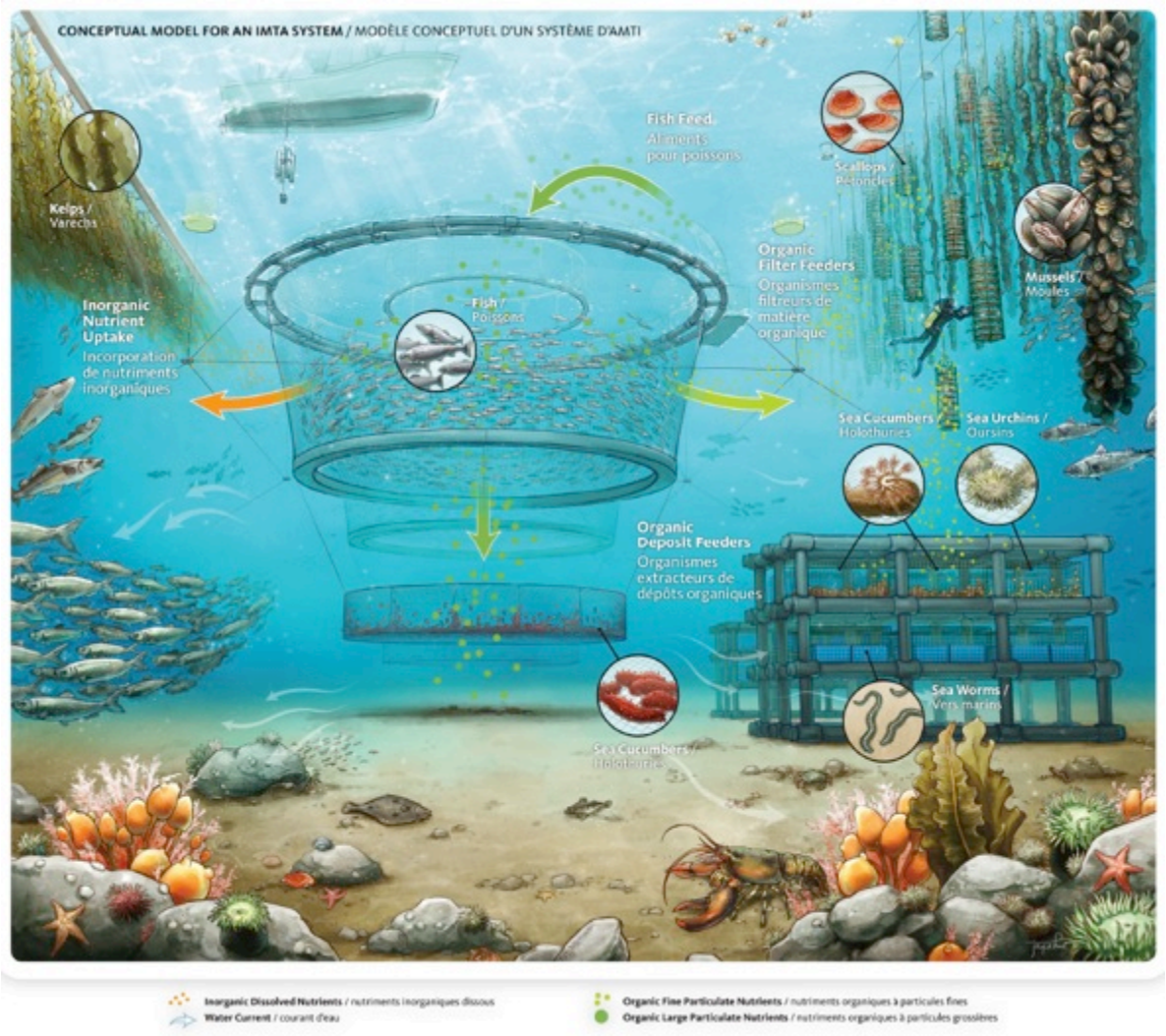

Figure 1.2: A conceptual model for an Integrated Multi-Trophic Aquaculture (IMTA) System, developed by scientists from the Canadian Integrated Multitrophic Aquaculture Network (CIMTAN). The dots and arrows represent the flow and uptake of inorganic dissolved (orange) and organic particulate (green) nutrients by added components. White arrows depict water currents. (Image from Canadian Department of Fisheries and Oceans: http://www.dfo-mpo.gc.ca/aquaculture/sci-res/imta-amti/images/imta-004.jpg)

1.4 Fish Pathogens and IMTA

One main theme to be investigated in the CIMTAN is how pathogens will interact with species added to such systems. One potential benefit of IMTA is the mitigation of pathogens spilling over from farms during disease outbreaks. For research specific to CIMTAN, filter-feeding shellfish are being investigated for their ability to ingest planktonic naupliar and copepodid stages of sea lice to potentially help reduce sea lice burdens on fish farms (Molloy et al., 2011; Webb et al., 2013; Bartsch et al., 2013). In fact, Bartsch and colleagues (2013) found that blue mussels and the Atlantic sea scallop (*Placopecten magellanicus*) can consume between 18-38% of sea lice (*Lepeophtheirus salmonis*) copepodids presented per hour, under laboratory conditions. Therefore, both filtering bivalve species may be suitable for low-level removal of sea lice copepodids over time (Bartsch et al., 2013). Field trials are currently underway to determine the feasibility of sea lice removal within an actual IMTA setting. If successful, this would be an added benefit of IMTA systems.

Overall, it appears that pathogens that are relatively environmentally fragile and easily filtered (*i.e.*, are of the correct size) are good candidates to be controlled via filtering bivalves in IMTA. For example, the infectious salmon anemia virus (ISAV) was removed and deactivated by filtering blue mussels within 24 hours (Skår & Mortensen, 2007; Molloy et al., 2014). Molloy and colleagues (2014) were able to detect ISAV virus within blue mussel digestive gland tissue with molecular techniques but the virus was not viabile within cell culture. There is evidence that blue mussels deactivate problematic bacterial species, such as the causative agent of bacterial kidney disease (*Renibacterium salmoninarum*; Paclibare et al., 1994). Additionally, cohabitation studies with the

bacterial pathogen *Francisella neatunesis*, filtered by mussels and exposed to Atlantic cod (*Gadus morhua*) did not yield clinical infection (Wangen et al., 2012).

However, IMTA systems will not be a panacea for diseases within aquaculture. Not all pathogens will be readily filtered, and there is evidence that certain pathogens bioaccumulate, and can even replicate within filtering bivalves. For instance, *Vibrio anguillarum* bacteria are easily ingested by bivalves and shed back into the environment (Pietrak et al., 2012). Both mussel feces and pseudofeces had high loads of *V. anguillarum* that proved infective to exposed cod (Pietrak et al., 2012). Additionally, infectious pancreatic necrosis virus (IPNV) appears to accumulate and replicate within filtering bivalves (Molloy et al., 2013). In the case of IPNV, viable virus was detected in mussel feces for up to 7 days post-depuration and in digestive gland tissues for at least 18 days, and caused infection in Atlantic salmon during cohabitation studies (Molloy et al., 2013). Even more concerning is how environmentally resistant pathogens, such as those that produce cysts, oocysts or spores, will interact with filtering bivalves. Bivalves such as mussels and oysters can act as bioindicators of the presence of human pathogens within drinking water sources (Willis et al., 2013). Filtering bivalves have been shown to filter and bioaccumulate pathogens such as *Toxoplasma gondii* oocysts (Arkush et al, 2003), *Cryptosporidium parvum* oocysts (Tamburrini & Pozio, 1999) and human-virulent microsporidia and *Enterococcus* spp. (Roslev et al., 2009).

1.4.1 Disease ecology and IMTA

As the aquaculture industry evolves, pathogens usually find a way to exploit new ecological niches revealed by any advancements. An especially important example is sea

lice, which consistently become resistant to any developed treatments (Costello, 2006). Just as there are pathogens that thrive in monoculture systems, there are several types of pathogens that may capitalize on trophic-based aquaculture systems. Overall, the types of pathogens that will take advantage of an IMTA system can be categorized in four ways. First, pathogens that can thrive in complex environments may be more problematic. In monoculture, biofouling is a regular problem that often has to be managed. However, when you add complexities such as mussels and seaweeds, it may prove difficult to manage the habitats and biofilms that these organisms support (*e.g.*, habitats support invertebrate intermediate hosts for several metazoans and protozoans). For example, *Neoparamoeba peruans*, the causative agent for amoebic gill disease, can proliferate within biofilms on fin-fish netting (Tan et al., 2002). Similarly, bryozoans are often found in biofilms and can carry the causative agent of proliferative kidney disease (*Tetracapsuloides bryosalmonae*; Bruno et al., 2006; Morris, 2012). Lastly, seaweeds and equipment for extractive species may provide ample habitat for fishes that are potential vectors for many pathogens.

Second, which is related to the first, pathogens that utilize intermediate hosts may emerge in an IMTA setting, especially metazoans such as digenean trematodes that use molluscs and invertebrates as intermediate and paratenic hosts. Additionally, myxosporidia such as *Ceratomyxa shasta*, reside within their intermediate host in complex habitats, such as freshwater mussels, before being exposed to their definitive host (Bartholemew et al., 1997). Third, pathogens that can infect multiple hosts would likely find multiple refuges within IMTA systems, such as IPNV and viral hemorrhagic septicemia virus (VHSV) (Gomez-Casado et al., 2011). Bacteria such as *Vibrio* spp. have

been shown to have complex interactions with their environment, utilizing several invertebrate and vertebrate species as reservoirs, vectors and hosts (Vessulli et al., 2002; Pruzzo et al., 2005; Beaz-Hidalgo et al., 2010). Lastly, which is relevant to the findings in this thesis, pathogens with environmentally stable life stages, and those that can withstand environmental variability will likely be problematic within IMTA systems. There are examples of environmentally stable viruses (*e.g.*, IPNV), bacteria (*e.g.*, Mycobacteria), metazoan and protozoan parasites (*e.g.*, trematodes, myxosporidia, microsporidia), all of which are environmentally stable or produce life stages that can withstand environmental variability (*i.e.*, spore, cyst and oocyst formation). Adding several trophic levels will increase the complexity of disease dynamics within these systems. Further knowledge on the complexities of disease ecology will help farm managers and fish health professionals anticipate the types of pathogens that may arise in an IMTA setting.

1.4.2 Loma salmonae as a model for environmentally stable pathogen interactions in IMTA systems

Of relevance to this thesis is the occurrence of environmentally resistant pathogens within a complex IMTA setting. Specifically; *L. salmonae* is a microsporidian parasite that causes gill infections in cultured salmonid fish. Briefly, *L. salmonae* matures in fish gills and infectious spores are released when necrotic cells lyse, allowing horizontal transmission to occur. Spores are environmentally resistant, and can persist in the environment (Shaw et al., 2000). In terms of the concepts described above, this thesis will utilize *L. salmonae* as a model for environmentally resistant pathogens that may exploit IMTA systems. As will be described below, a reliable *Loma salmonae*-rainbow trout

(LS-RT) disease model has been in use for over 15 years (Speare et al., 1998a). It will be manipulated to elucidate interactions between blue mussels and rainbow trout as a proxy of how an environmentally stable pathogen may behave within an IMTA system.

1.5 Microsporidian Parasites

Microsporidia are obligate intracellular parasites that belong to the Fungi kingdom (James et al., 2013). They are highly diverse pathogens, with over 1200 described species that infect all levels of invertebrate and vertebrate life (Sprague et al., 1992). The microsporidian genome is extremely truncated to accomodate its lifestyle as an obligate intracellular pathogen (Keeling et al., 2010; Keeling and Corradi, 2011; Peyretaillade et al., 2015). Microsporidian cells do not contain typical mitochondria or Golgi, and are missing peroxisomes entirely (Vávra & Lukeš, 2013). A highly reduced mitochondria called a mitosome is present but it does not have a genome, and cannot support the electron transport chain or Krebs cycle (Esser et al., 2001). The Golgi apparatus is not stacked, but occurs as a mass of vesicular tubules with direct connection to the endoplasmic reticulum (ER) (Beznoussenko et al., 2007). The reduced Golgi is present throughout its life cycle and is particularly apparent during sporogony (Esser et al., 2001). The reduced organelles make microspordia highly reliant on host cells for respiration and membrane manufacturing (Corradi & Selman, 2013). Importantly, microsporidians have transport proteins to import adenosine triphosphate (ATP) from the host cytoplasm to support their reduced metabolism (Weidner & Trager, 1973; Tsaousis et al., 2008).

Given the diversity of microsporidia life cycles can vary greatly, but generally consist of a dispersive spore stage and an intracellular replicative stage. The infectious

particle is the spore. Spores are generally small (2-7 μm by 1.5 – 5 μm; Bigliardi & Sacchi, 2001) and contain a complex electron-dense exospore and electron-lucent endospore wall (Frixione et al., 1997). The thick endospore is primarily made up of chitin (Erickson & Blanquet, 1969; Vávra, J., & Lukeš, 2013). The thickness and stability of the exo- and endospore allow for environmental stability and to create pressure differences that lead to spore discharge (Frixione et al., 1997). Underneath the spore wall is a highly electron-dense plasma membrane surrounding the spore contents: the sporoplasm and extrusion apparatus. The sporoplasm is the infectious material and can contain either one or two diplokaryon nuclei along with a cytoplasm filled with ribosomes (Sprague et al., 1992; Keeling & Fast, 2002). The extrusion apparatus consists of the polar filament (*i.e.*, polar tube), the anchoring-disc, the polarplast, and the posterior vacuole. The polar tube is made up of a straight, anterior portion that is surrounded by the anterior lamellar polarplast and is attached to the inside of the spore by the anchoring disc. The posterior coiled region is the most prominent feature of microsporidian spores, and is often used for identification via the number of times it coils around the sporoplasm (Sprague et al., 1992; Wittner & Weiss, 1999). Polar tubes can range from 50 to 100 μm in length and 0.1 to 0.15 μm in diameter (Frixione et al., 1992). The anterior lamellar polarplast is an arrangment of flattened membranes that surround the straight portion of the polar tube. The vesicular polarplast consists of tubular structures located mid-spore. Both polarplast membranes are continuous with the polar tube membrane and are rich in carbohydrates (Canning & Lom, 1986). The posterior vacuole is a membrane-bound organelle that is prone to swelling from changes in osmotic pressure, ultimately leading to polar tube discharge (Undeen & Frixion, 1990). (Figure 1.3)

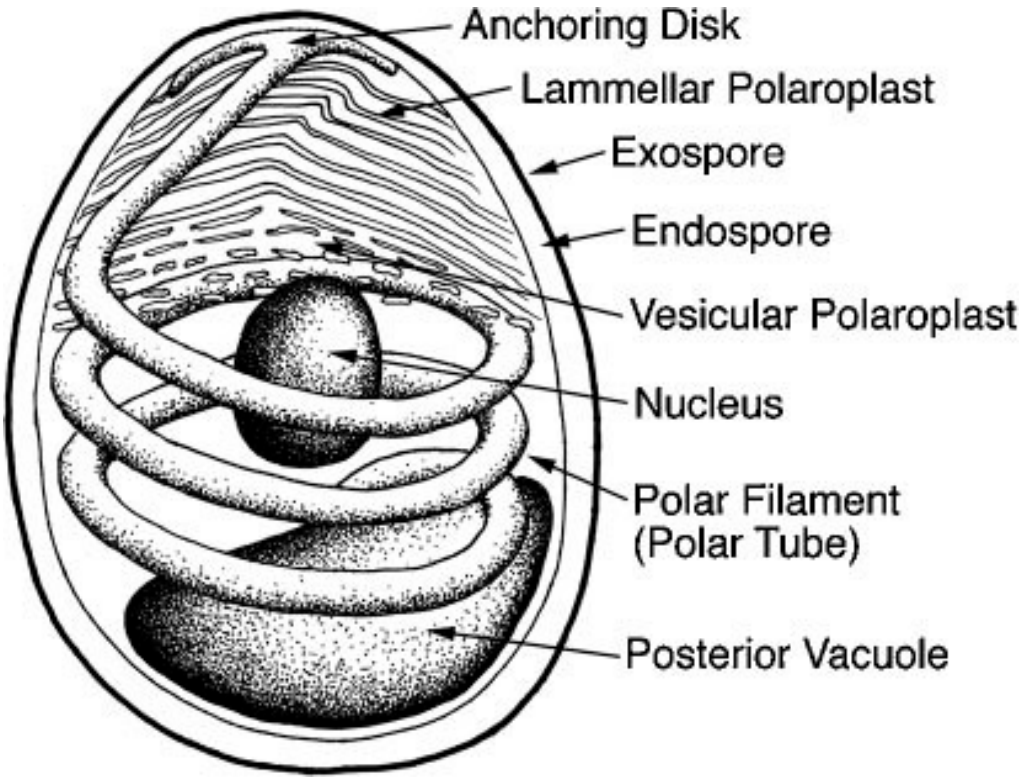

Figure 1.3: Diagram outlining the common components of microsporidian spores, as labeled. From Keeling and Fast, 2002.

Spores are given environmental and chemical signals to germinate and release their sporoplasm. Ideally, the result of germination is the injection of sporoplasm into a host cell. Spores are initially activated by many stimuli that elicit an increase in osmotic pressure within the spore (Bigliardi & Sacchi, 2001). Stimuli can include: pH shifts, dehydration then rehydration, hyperosmotic surroundings, presence of cations or anions, exposure to ultraviolet light, etc. (Keohane & Weiss, 1999; Xu & Weiss, 2005). Often the signal is a pH shift within the host gastrointestinal system after ingestion of a spore. When the correct environmental signal occurs, the osmotic pressure inside the spore increases, resulting in swelling of the polarplast which initiates polar tube eversion

(Undeen, 1990; Frixione et al., 1992). The posterior vacuole also expands, acting to push the sporoplasm through the everted polar tube (Weidner et al., 1984). If the sporoplasm is directly injected into a host cell it is enveloped by a plasma membrane formed by the polarplast or host cell membrane (Bigliardi & Sacchi, 2001; Xu & Weiss, 2005). It appears that spore surface proteins are involved in spore aherance to host cells which may influence tissue tropism and regulate phagocytosis and infection (Leonard, 2013). The polar tube can inject sporoplasm into host cell cytoplasm if the spore has been phagocytozed to escape the digestive phagolysosome (Franzen, 2004). Spores in lysosomal compartments are rapidly digested, but injected sporoplasms appear to evade the formation of phagolysosomes (Franzen et al., 2005). Either method of invasion ensures protection from recognition and reaction by host immune mechansisms (Franzen et al., 2005). (Figure 1.4)

Once inside a host cell, microsporidians can develop in direct contact with host cytoplasm, or they can develop within a membrane (*i.e.,* the parasitophorous vacuole, PV) that can be derived from the host, the parasite or a combination of the two (Franzen et al., 2005). In all cases, the sporoplasm proliferates in a process called merogony. Division of meronts can occur via binary fission, or karyokinesis occurs without cytokinesis, producing multinucleate cells (Bigliardi & Sacchi, 2001). During merogony the parasite is highly dependent on host cell machinery and are surrounded by host organelles, namely the ER and mitochondria (Vávra & Larsson, 1999). There is evidence that during meorgony, the parasite utlizes the host ER-related autophagic mechanisms (Lovy et al. 2007). The parasite can also completely transform host cells into highly enlarged xenoparasitc complexes termed xenomas to promote unfettered growth and

proliferation, hidden from host immune defenses (Weissenberg, 1976; Canning et al., 1982). (Figure 1.4)

Once merogony is complete, sporogony begins to produce sporoblasts, which then become mature spores (Bigliardi & Sacchi, 2001). Nuclei within meronts separate and the plasma membrane thickens and becomes more electron-dense as proteins are deposited (Vávra & Larsson, 1999). The ER and Golgi become more ordered and prominant, as do ribosomes, increasing cytoplasmic density (Vávra & Larsson, 1999). Divided sporonts become sporoblasts and it is during this stage that the polar filament and other elements of the extrusion apparatus develop (Keeling & Fast, 2002). The cells then decrease in size and the chitinous endospore layer forms (Keeling & Fast, 2002). Once spores mature, the cell usually lyses and spores are released. Released spores can then go on to autoinfect other nearby cells (Solter & Maddox, 1998), or are released into the environment where they can infect other nearby hosts (Keeling & Fast, 2002). Spores can also survive when hosts are not immediately present through the environmental resistance of the spore, through the use of alternate host species (Becnel & Andreadis, 1999) and the presence of spores in host gonadal material for vertical transmission (Hunt et al., 1972; Canning et al., 1985; Dunn & Smith, 2001). (Figure 1.4)

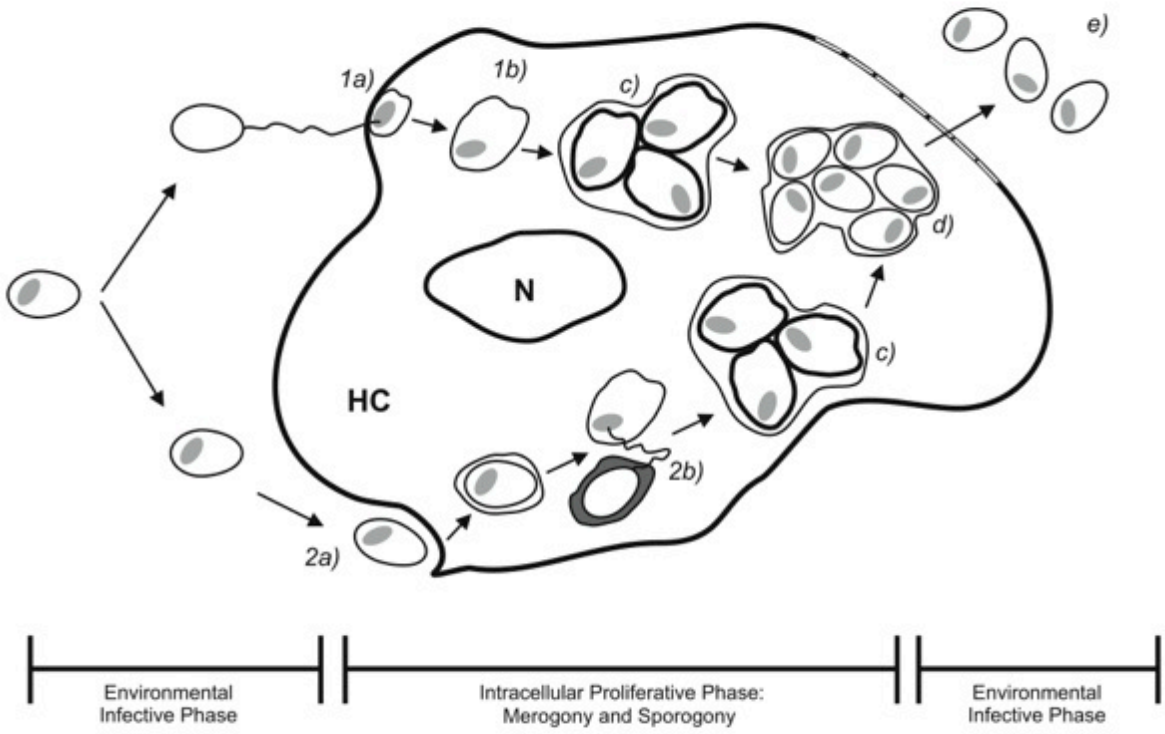

Figure 1.4: The generalized life cycles of microsporidia. Infectious spores in the environment can gain access to cells in 2 ways: Spores germinate and directly inject their sporoplasm into a cell **(1a)** or are phagocytized **(2a)** and evade lysozomal degradation **(2b)** to gain entry into the host cell. Once inside the cell, merogony typically occurs in direct contact with host cytoplasm **(1b)**. Sporogony may **(c)** or may not occur with a membrane. Spore maturation occurs **(d)**, and spores are released into the environment when the host cell dies **(e)**. **HC** host cell, **N** nucleus of host cell. From: Monaghan, 2011.

The pathology associated with microsporidian infections is commonly due to a high parasite burden, immunodeficiency of the host, or an overactive inflammatory response to spores within tissues. Infections are usually sub-clinical but in certain instances are devastating. For example, *Nosema apis* is highly pathogenic to colony-forming honey bees due to widespread destruction of host cells when mature spores are released. Spores can mature within 48 hours and are quickly disseminated to other colony members via feces (de Graaf et al., 1994). In humans, *Encephalitozoon cuniculi* produces large numbers of spores in the gut (Canning & Lom, 1986). The parasite also causes chronic infection in the kidney, liver and brain, which is usually subclinical (Yachnis et al., 1996). However, in immunocompromised hosts, such as humans with AIDS, the infection becomes opportunistic and disseminates widely, causing severe pathology and death (Dunn & Smith, 2001). In fish, microsporidia such as *Pleistophora* spp., infect the gut and then disseminates to muscle tissue and can cause widespread tissue damage (Canning & Nicholas, 1980). Alternatively, fish-infecting microsporidia such as *Glugea* spp. and *Loma* spp. form slow-growing xenomas. The pathology from xeonmas usually results in a local inflammatory response that can cause devastating pathology in heavy infections (Dyková & Lom, 1980; Lovy et al., 2007).

1.5.1 Microsporidia in fish

According to Lom and Nilsen (2003), there are 156 recorded species of microsporidians that infect fish, within 14 genera (See Lom, 2002 for a complete catalogue). As with other microsporidians, most infections are sub-clinical and ubiquitous. However, when microsporidiosis occurs within a high-density culture scenario, it can be quite devastating and may even limit the culture of a specific fish (*e.g.*,

Loma morhua and cultured Atlantic cod *Gadus morhua*; Khan, 2005). There are no widely effective and/or approved drugs that can treat microsporidian infections and thus constant mortality can occur, along with growth-rate reduction as reported for salmonids infected with *L. salmonae* (Speare et al., 1998b). Additionally, chronic microsporidiosis caused by *Pseudoloma neutrophilia* within laboratory-reared zebrafish (*Danio rerio*) is widely prevalent within research facilities (>74%; Murray et al., 2011) which has implication for a wide variety of research, including human medical research (Sanders et al., 2012).

In fish, infective spores are usually transmitted horizontally by ingestion following exposure in the water column. After ingestion, spores likely germinate within the gut and enter epithelial cells. Parasites can develop at the site of infection but are often transported to other tissues, presumably by circulating leucocytes (Shaw et al., 1999). There is also strong evidence suggesting that infections can be vertically transmitted through infected eggs (*e.g., P. neurophilia* in zebrafish; Sanders et al., 2013).

One special feature of fish microsporidians is the majority of species develop into xenomas. In non-xenoma-forming microsporidians (*e.g., Nucleospora*, *Pleistophora*, *Kabatana*, and *Heterosporis* spp.), the developmental stages are located directly in the cytoplasm without any specific boundary (Lom & Nilsen, 2003). Xenoma-forming microsporidnas often have a higher degree of host specificity (Lom & Dyková, 2005). Within xenomas, the cell and its nucleus become hypertrophic, apparently changing physically and biochemically in order to protect and support the developing parasite (Lom & Dyková, 2005). Organisation of the xenoma differs by species, with some (*e.g., Nosemoides spp.*) being covered by a wall formed by the plasmalemma and surrounding

fibroblasts (Faye et al., 1996), and others (*e.g., Tetramica* spp.) being covered by microvillous processes (Matthews & Matthews, 1980). Distrubution of developing stages can be random (*e.g., Loma spp*.), or organized by stage (*e.g., Glugea* spp., and *Spraguea* spp.) (Lom & Nilsen, 2003).

Meronts in fish microsporidia are usually in direct contact with the cytoplasm and are either uninucleate and divide by binary fission (*e.g., Nucleospora salmonis*) (Desportes-Livage et al., 1996), or grow into multinucleate plasmodia and divide by plasmotomy or by multiple fission (*e.g.,* in *Glugea* spp.) (Canning, 1982; Lom & Nilsen, 2003). Sporogony is quite variable among species, but is always marked by the formation of a thick dense coat, usually within a vacuole of host or parasite origin (Lom & Nilsen, 2003). Sporoblasts take the usual form, as do spores, although lots of species-specific variation exists in appearance and structure of the exo- and endo-spore. Some species (*e.g., Pleistophora* and *Heterosporis* spp.) even produce macro- and microspores that differ in size and polar-tube turns (Lom & Nilsen, 2003).

In terms of resistance mechanisms, spores are readily phagocytized and destroyed by macrophages (Canning & Lom, 1986). Soluble plasma factors such as complement and lectins may help increase phagocytic activity (Shaw et al., 2001). However, neutrophils may or may not destroy phagocytized spores, and have been implicated in housing infection (Lovy et al., 2007). Dendritic-like cells are theorized to act as antigen-presenting cells during infection (Lovy et al., 2007). Fish develop an apparently weak humoral response against microsporidians by generating specific antibodies against parasite antigens (Leiro et al., 1993; Sánchez et al., 2001). Cellular immunity (*i.e., via* T-lymphocytes) is thought to be more important in protection against microsporidia in fish

(Rodriguez-Tovar et al., 2006, 2011). Most drug treatments for microsporidians are ineffective (Speare et al., 1998a; Shaw et al., 1999). Immunostimulant use and vaccination appear to be the most promising way to manage microsporidian infections (Rodruiguez-Tovar et al., 2011; Guselle et al., 2010; Harkness et al., 2013).

1.5.2 Genus Loma

Several species within the *Loma* genus can cause serious disease in wild and cultured fishes, for example *L. morhua* [Nemeczek, 1911] in Atlantic cod *G. morhua* (Morrison & Sprague, 1981), *L. salmonae* [Putz, Hoffman and Dunbar, 1965] in salmon and trout spp. (Morrison & Sprague, 1981). Microsporidians in the genus *Loma* are typically recognized by the formation of xenomas in the gills or other vascularized tissues. Developmental stages within xenomas are irregularly distributed. Xenomas contain a hypertrophic nucleus and often have a thick layer of granular, amorphous substance covering a plasmalemma (Lom & Pekkarinen, 1999; Lom & Nilsen, 2003). Multinucleate meronts divide by binary fission into uninucleate cells, or multinucleate plasmodia in direct contact with the host cytoplasm (Morrision & Sprague, 1981; Lom & Nilsen, 2003). Sporogony occurs within a membrane origining from the parasite PV which usually contains 1-8 spores and type II tubules surround the spores during development (Canning & Lom, 1986; Lowman et al., 1999).

There are currently 19 reported species within *Loma* (See Table 5 in Brown et al., 2010a for details), but they vary considerably and were likely combined because of a lack of genetic analysis (Lom, 2002; Lom & Nilsen, 2003). Additionally, *Loma* species are usually described based on host species, spore and xenoma size which show a large degreee of overlap and may not describe the actual species designation genetically

(Brown et al., 2010a). Current genetic analyses have clarified species relationships but more research is needed (Brown et al., 2010a).

1.6 *Loma salmonae*

1.6.1 Microsporidial gill disease of salmonids

L. salmonae (Putz, Hoffman, & Dunbar, 1965) is the causative agent of microsporidial gill disease of salmon (MGDS). MGDS was first described in 1981 during an outbreak of gill disease in rainbow trout at a hatchery in California (Morrison & Sprague, 1981). During the mid-1980s it was discovered that *L. salmonae* was a contributor to fish losses in salmonid aquaculture on the west coast of Canada. For example, between 1984-1986, *L. salmonae* was determined to be the major contributor to mortality and gill disease in coho salmon (*Oncorhynchus kisutch*) culture in Washington State, USA (Kent et al., 1989; Speare et al., 1989). Infections were evident in the gills of some fish before seawater entry, but significant pathological changes in the gills occurred after transfer into seawater cages (Kent et al., 1989). Systemic infections were also observed in farmed rainbow trout in Scotland (Bruno et al., 1995). Low mortality occurred during this outbreak and fish had xenomas associated with blood vessels and pillar cells of gill lamellae and the secondary lamellae were hyperplastic (Bruno et al., 1995). Infections have also been seen in freshwater hatcheries (e.g., Rainbow trout; Markey et al., 1994).

Although infections are usually associated with a culture setting, *L. salmonae* has recently been identified in wild Sockeye salmon (*O. nerka*) kills in Alaska, USA (Weber et al., 2013). Although many fish have *L. salmonae* present in their gills when arriving on spawning grounds, they rarely cause disease that can influence population numbers.

However, Sockeye salmon in Alaska are experiencing major environmental challenges and popultion numbers are dwindling. Smaller returning year classes, along with increased stressors, seems to have increased disease impacts on populations with low genetic diversity (Weber et al., 2013). Although mapping the impact of *L. salmonae* in wild fish is difficult, these events will likely become more commonplace.

MGDS is especially problematic in Chinook salmon (*O. tshawytscha*) culture in British Columbia, Canada (Constantine, 1999). *L. salmonae* is seasonally endemic in farmed Chinook salmon, and causes increased mortality during their second year in ocean net pens (Lovy et al., 2008). It is likely that wild fish act as a reservoir, as infection can occur in all Pacific salmonid species (Kent et al., 1998; Kent, 2000). During infection, xenomas alter gill architechture and the inflammatory response is associated with fibrinoid arteritis, periartiritis, and hyperplasia of overlying epithelium (Hauck, 1984, Kent et al., 1989, Speare et al., 1989, Kent et al., 1995). The tissue damage and inflammatory response is associated with suppressed growth rates, decreased respiration, increased metabolic costs, and ionic disturbances - all which are problematic in a farm setting (Speare et al., 1998b; Powell et al., 2005; 2006). Outbreaks of disease in net-pen reared chinook are typically observed in the late summer, which coincides with the optimal water temperature for MGDS infection (Speare et al., 1999; Beaman et al., 1999). MGDS remains a seasonal problem with chinook farms, and treatments are often ineffective, given the intracellular nature of the parasite.

1.6.2 Characteristics of L. salmonae

The mature *L. salmonae* spore is approximately 4 µm long and 2 µm wide (Morrison & Sprague, 1983). Two variants exist in net-pen reared Chinook salmon: the

typical (OA; 17 polar tube coils) variant has host specificity for salmonids and the atypical (SV; 20 polar tube coils) variant is specific to brook trout, *Salvelinus fontinalis* (Sánchez et al., 2001; Lovy et al., 2004). Genetic analysis of *L. salmonae* and related species have confirmed that the SV variant is actually a distinct but related species and discussion related to *L. salmonae* henceforth refers to the OA strain (Brown et al., 2010b). *L. salmonae* is xenoma-forming and appears to favour endothelial cells, leucocytes and pillar cells in the gills (Rodriguez-Tovar et al., 2002).

Horizontal transmission usually occurs after an infective spore is ingested by a susceptible salmonid (Shaw et al., 1998; Sánchez et al., 2001). Vertical transmission is also thought to occur. Spores are commonly found in the ovaries of mature Chinook salmon, and passage of spores likely occurs via ovarian fluid (Docker et al., 1997). Germination is likely triggered by the pH shift of passage from the acidic stomach to the alkaline upper intestinal tract. It appears that the polar tube directly injects the sporoplasm into the lamina propria of the gut, with the presence of *L. salmonae* observed via *in situ* hybridization within 12 hours (Sánchez et al., 2001). However, the parasite does not remain in the gut for long, nor is it the major site for xenoma development and parasite maturation. Although it has not been confirmed, it is likely that circulating leucocytes transfer the parasite to the heart within 2 days (Sánchez et al., 2000). The parasite appears to undergo merogony within the heart, but is not the optimal tissue type for maturation (Sánchez et al., 2001). In heavy infections in Chinook salmon, xenomas may mature within the heart tissue, but this is rare in most cases. The parasite reaches the gills 2 weeks post infection and it is here that final maturation usually occurs (Sánchez et al., 2000). Again, it is likely that developing parasites reach the gill via circulating

leucocytes, although this has not been observed *in vivo* (Sánchez, 2001). By 2 weeks post infection, DNA of developing parasites have been detected by *in situ* hybridization, but xenomas cannot be visually detected until 4 weeks after exposure (Speare et al., 1998b).

Once the parasite arrives in the gill, the cell containing the parasite either becomes trapped within the fine vessel system, or the parasite and cell is phagocytized by a more permissive cell within the gills, such as a pillar cell (Rodriguez-Tovar et al., 2002). Once the parasite is in its optimal permissive cell type or optimal region for development, xenoma formation occurs and the developing meronts undergo sporogony and finally develop into mature spores. Lovy and colleagues (2006) hypothesize, that the merogonial stage is surrounded by host ER, forming a host-derived PV. The ER surrounding the merogonial stage, along with the plasmalemma surrounding the meront, gives the appearance of 3 membranes (assessed via TEM; Lovy et al., 2006). The inner ER remains in close association with the merogonial plasmalemma to eventually form the double membrane of the sporogonial wall. The process of ER surrounding a cytoplasmic element resembles cellular autophagy, which is used by host cells to degrade excess, spent or injured organelles (Yoshimori, 2004). Usually the cisternae of ER surround the element to be degraded to form an autophagosome, which then fuses with lysozomes forming autophagolysozomes to allow degradation (Yoshimori, 2004). Several pathogens (*e.g.*, *Brucella abortus*) utilize this function to survive within host cells allowing them to avoid host cell immune mechanisms and obtain nutrients for development (Dorn et al., 2002). Developing within the lumen of the ER can provide ample access to proteins and enzymes that would allow protein development, such as for the formation of polar tube and spore wall elements. It is possible that development of the vacuole is in response to

the presence of sporoplasm within the cytoplasm, and then the parasite utilizes the autophagosome for replicative purposes (Lovy et al., 2006).

The xenoma can be quite large, often reaching several mm (Rodriguez-Tovar et al., 2002). The developing parasite within xenomas take about 1-4 weeks to produce mature spores. Mature xenomas become encapsulated by fibroblasts and collagen, with reinforcing desmosomes between fibroblasts (Lovy et al., 2007). Once the xenomas enlarge further they can be observed as white cysts on gill filaments with the naked eye or under a dissecting microscope (Kent et al., 1995). Under optimal temperature conditions the infection takes about 6 weeks from time of infection to maturation and rupture of xenomas. The inflammatory mechanisms involved in infection have been well documented by Lovy and colleagues (2007). In late stages of xenoma maturation, host inflammatory cells start surrounding it, likely recruited by antigenic signals across the xenoma membranes and autoinfecting spores through the xenoma wall (Lovy et al., 2007). Eventually, phagocytic cells infiltrate the xenoma and are thought to begin clearing spores, although spore degradation within neutrophils has not been observed (Lovy et al., 2007). Neutrophil-coordinated infiltration leads to destruction of the gill filament and lamellar basement membrane, allowing spore access to the environment (Lovy et al., 2007). It also appears that neutrophils begin to form multi-nucleated giant cells for extracellular killing of spores within tissue during xenoma degradation (Lovy et al., 2007). Thrombocytes are also active in the inflammatory response, and are recruited to sites of vascular injury to form thrombi near damaged endothelium (Lovy et al., 2007). Dendritic-like cells are also present in the inflammatory response, and are presumably associated with antigen-presentaton for the cellular immune response (Lovy et al., 2007).

Neovascularization also occurs, allowing for vascular remodelling following the inflammatory response (Lovy et al., 2007).

The permissive temperature range for infection is between 9 and 20°C (Speare et al., 1998a; Beaman et al., 1999). The optimal temperature appears to be around 16°C, but there is no difference in xenoma numbers in fish exposed within the above range (Speare et al., 1998a; Beaman et al., 1999). Xenoma development is absent outside of this range, and xenomas fail to develop even if fish are moved to the permissive temperature range (Beaman et al., 1999). Utilizing *in situ* hybridization techniques, Sánchez et al. (2000) characterized the normal progression of infection at 15°C as described above. Parasite DNA was detected in the hearts of rainbow trout 3 days following exposure, in the gill by 2 weeks and xenomas were developing by week 4 (Sánchez et al., 2000). For fish held in 5°C water, the parasite did not reach the heart until 7 days, but was also in the gill at 7 days even though xenomas failed to develop and parasite DNA could not be detected by 4 weeks (Sánchez et al., 2000). Alternatively, for fish held and exposed at 21°C, parasite DNA was detected in the heart, spleen and gills by 3 days (Sánchez et al., 2000). No xenomas developed for fish held at 21°C and DNA was no longer detected by week 2 (Sánchez et al., 2000). Therefore, temperature appears to regulate how the parasite is transmitted within the fish (Sánchez et al., 2000). Immune functions appear to work at faster rates in higher temperatures so this result is intuitive (Roberts, 2012).

During heavy infections, spores appear to autoinfect surrounding tissues (Shaw et al., 1998). This is especially common in Chinook salmon, where infections can persist for up to 20 weeks post-exposure (Kent et al., 1998). In more moderate infections, fish mount an immune response to clear infection by 10 weeks post-exposure (Speare et al.,

1998a). However, free spores have been detected within kidney interstitium several months after xenoma clearance (Kent et al., 1998). Once recovery from infection has occurred, salmonids show strong resistance to re-infection (Speare et al., 1998a; Kent et al., 1998). Additionally, fish infected outside their optimal temperature range are protected from re-infection (Beaman et al., 1999). In vaccinated fish, the cell-mediated immune response becomes protective 4 weeks after exposure. Specifically, it appears that in vaccinated fish, prebranchial merogonic life stages in the subendocardial macrophages are cleared. It is not just *L. salmonae* spores that confer protection for MGDS, it appears that other fish-infecting microsporidians (*e.g., G. anomala* and *G. hertwigi*) can act as effective vaccines (Harkness et al., 2013).

Treatments for *L. salmonae* and MGDS are genereally not useful. Clinical disease is associated with the inflammatory response relating to xenoma rupture, and thus it is difficult to ascertain when proper treatment should be applied. The inflammatory response can be quite extreme in heavy infections, causing respiratory distress due to damaged gill tissue exacerbated by inflammatory cytokines. Gills can become quite hypertrophic with lammellar fusion and subsequent respiratory distress. Anti-parasitic compounds can be useful, but only when applied prior to xenoma onset (Becker et al., 2002).

1.7 *Loma salmonae*-Rainbow Trout (LS-RT) Disease Model

Much of the information relating to infection with *L. salmonae* has been revealed by laboratory studies. A reliable LS-RT disease model has been in use since 1998 (Speare et al., 1998a). MGDS can be induced by: feeding naïve fish infectious tissue, injecting purified spores intraperitoneally (IP), anal gavage, intramuscular and intravascular

injection, co-habitation, and through exposure to contaminated water (Shaw et al., 1998; Becker & Speare, 2004). The LS-RT model was developed when MGDS first became a concern in farmed salmonids. Efforts towards completing the parasitic life cycle within laboratory fish under controlled settings has enabled increased understanding of the pathogen and mechanisms for its control in the field. *L. salmonae* can infect many *Oncorhynchus* and *Salvelinus* spp. but not Atlantic salmon. Infections are especially problematic in Chinook salmon, which develop the most severe pathology (Ramsay et al., 2002). The experimental model utilizing rainbow trout as the sequelae of infection is similar, but pathology is less severe (*i.e.,* for welfare reasons) (Kent and Speare, 2005). Generally infection is induced by exposure to spores *per os* or via IP injection. Fish are maintained in tanks at 15°C for 6 weeks, or until the desired maturity of infection is reached.

1.8 Improving the Disease Model: Using Monoclonal Antibodies to Identify Spores

Generally, microsporidians are definitively identified using TEM and/or PCR techniques (Didier et al., 1996). Live spores can be non-specifically identified using histochemical techniques such as chitin-staining fluorochromes (Didier et al., 1996). Techniques using polyclonal and monoclonal antibodies (MAbs) have been clinically useful for identifying microsporidians infective to humans, such as *E. bieneusi* for example, in stool samples of infected patients (Accocenerry et al., 1999). Components of the chitin-containing endospore are common antigens that cause antibody reactions in mice and rabbits immunized with microsporidia (Accoceberry et al., 1999). The MAb techniques developed to identify spores in human stool samples are highly specific when compared to TEM, PCR and different staining techniques (Accoceberry et al., 1999).

Diagnoses can be made in subclinical infections where few spores are present (Accoceberry et al., 1999). Utilizing MAbs as an identification tool in a diagnostic setting is ideal, given the time-to-identification which compared to other techniques (*e.g.*, PCR and TEM) is very short (Accoceberry et al., 1999). MAbs are also useful for the isolation and purification of spores using chromatography or immnomagnetic separation.

1.9 Improving the Disease Model: Microsporidians in Cell Culture

The RT-LS model has been very useful in understanding *L. salmonae* and MGDS. However there are some limitations. Since whole spores can elicit resistance in fish it has been suggested that this disease model could be utilized in the development of a vaccine for use in cultured fish (Harkness et al., 2013). However, with principles of animal reduction in mind, it is prudent to investigate alternative methods of spore production rather than rely on the use of live fish for the mass production of spores. Additionally, xenoma development has not been wholly characterized which has limited investigations into stopping development therapeutically. Lastly, drug developments have been limited since there are restrictions for *in vivo* treatment testing.

One way to work around these limitations would be to grow *L. salmonae in vitro*. Cell culture techniques are utilized in many disease models and it presents an interesting opportunity for spore development using *in vitro* techniques. Cell culture of microsporidian parasites have been in development since the early 1950s and have become quite useful for the production of spores for use in scientific study of pathogens, drug screening and spore production for various reasons (Visvesvara, 2002; Monaghan, 2011).

1.9.1 Insect microsporidians in cell culture

N. bombycis was the first microsporidian to be cultured while the silkworm industry was experiencing a collapse due to widespread microsporidiosis (Trager, 1937). As insect cell lines were rapidly developed in the 1960's, microsporidia became reliably culturable. Investigations into life cycles and developing spores for research and commericial biocontrol applications are common for insect-infecting cultures (Jaronski, 1984; Becnel et al., 2005). The life cycle of intracellular proliferation was discovered using this technique and drug screening has been successful. For bees and colony collapse syndrome, *in vitro* techniques have been extremely useful in identifying the infectious agent and for drug screening efforts. In another interesting application of cell culture and microsporidians, spore propagation occurs at commerical levels for the biocontrol of locust populations (Sokolova et al., 2003).

1.9.2 Human microsporidians in cell culture

Cell culture techniques for human-infecting microsporidians is extremely important. Results vary by species, but microsporidians are often cultured to amplify spore numbers for identification and drug screening applications (Visvesvara, 2002). Cultures have also provided invaluble information (*e.g.*, infection processes, life cycle clarification) relating to the biology of microsporidians (Belkorchia et al., 2008).

1.9.3 Fish microsporidians in cell culture

Although microsporidians are of great economic concern in fish, culturing fish-infecting microsporidians has been much less successful than for human and insect-infecting microsporidians. There are more than 150 species of fish microsporidians, many

of economic concern, but only 6 of them have been successfully cultured *in vivo* (Monaghan, 2011; Saleh et al., 2014). Primary culture has been quite useful, but is generally short-lived and highly variable (Monaghan, 2011). Perhaps the most useful primary culture model has been with *Nucleospora salmonis*, which allowed study into the life cycle of the parasite (Desportes-Livage et al., 1996).

It is unknown why there are so many difficulties surrounding the culture of fish-infecting microsporidians but it may be that fish-infecting microsporidians tend to be more host specific than others. One reason for this increased susceptibility may be that that the majority of fish-infecting microsporidians are xenoma forming, which may require multiple cell types for complete development (Lom & Dyková, 2005). It is unclear whether xenoma-forming microsporidians can be successfully cultured *in vitro*.

1.10 Thesis Objectives

Overall, this thesis was designed to determine whether the LS-RT disease model could be manipulated to include filtering bivalves as a component to determine how an environmentally resistant pathogen, *L. salmonae* would behave within complex IMTA systems. Through the addition of blue mussels to the LS-RT disease model it was discovered that a more reliable way to correctly identify *L. salmonae* spores in mussel tissues, feces, and pseudofeces had to be developed. Therefore a MAb-PI spore identification and viability system was developed to ensure that spores could be reliably identified. Lastly, experiments in this thesis required high spore numbers for experimentation. Cell culture techniques were developed to try and provide a mechanism to amplify spore numbers *in vitro*, while keeping fish use to a minimum.

***Aim 1*:** Add blue mussels to the existing LS-RT disease model to determine how environmentally resistant pathogens behave within complex IMTA systems.

Working hypothesis: Blue mussels will efficiently filter *L. salmonae* spores in laboratory conditions.

Rationale: *L. salmonae* spores fall within the optimal size range of particles ingested by blue mussels and thus it is theorized that they will effectively filter spores from the water column.

***Aim 2*:** Be able to observe the passage of *L. salmonae* spores within blue mussels to determine how they interact with the parasite

Working hypothesis: Spores will be easily observed within the tissues, feces and pseudofeces of blue mussels in such a way that spore passage will be detectable using laboratory techniques.

Rationale: An existing MAb, specific to *L. salmonae* spores should prove useful in detecting spores in mussel tissue, feces and pseudofeces to determine the fate of such spores.

***Aim 3*:** Develop a reliable *in vitro* method to confidently identify *L. salmonae* spores and determine their viability

Working hypothesis: The viability of spores corresponds to the presence of intact spore membranes.

Rationale: Spores will be easily and reliably identified using an existing MAb specific to *L. salmonae* spores. The addition of PI will allow us to determine viability that

corresponds to *in vivo* results, using the LS-RT disease model.

***Aim 4*:** Propagate large number of *L. salmonae* spores within cell culture

Working hypothesis: As with other microsporidians, *L. salmonae* will be easily propagated within appropriate cell culture media.

Rationale: Many microsporidians have been propagated using cell culture techniques. Some are even commercially propagated for the use of pest biocontrol. Given the plethora of literature relating to microsporidia culture, it should be possible to culture *L. salmonae*.

***Aim 5*:** Determine the best way to store viable *L. salmonae* spores for an extended period of time

Working hypothesis: L. salmonae spores will be successfully stored within a phagocytic cell line, *Aedes albopicus*, for future viability of the LS-RT disease model.

Rationale: *L. salmonae* spores are not viable following conventional freezing or placement within liquid nitrogen. It appears that spores of this species require a more protective cryopreservation technique to remain viable.

1.11 References

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Chapter 2.0 MICROSPORIDIAL SPORE (*LOMA SALMONAE*) VIABILITY FOLLOWING EXPOSURE TO BLUE MUSSELS (*MYTILUS EDULIS*)[1]

2.1 Abstract

Integrated multi-trophic aquaculture (IMTA) utilizes waste produced by fin-fish culture for the growth of organic-extractive species with complementary trophic functioning. However, the addition of extractive species (*e.g.*, bivalves) may increase the complexity of disease dynamics within IMTA systems. A series of laboratory *in vivo* studies were devised to probe the transfer and fate of microsporidial spores of the salmon pathogen *Loma salmonae* to the blue mussel (*Mytilus edulis*). An *in vivo* rainbow trout (*Oncorhynchus mykiss*) branchial xenoma expression model was used to detect the presence of viable microsporidial spores within aquarium-held blue mussels. Viscera from mussels exposed to spores for 4 hours were fed to trout that subsequently developed xenomas within their gills 6 weeks post-infection. However, mussel-fed fish yielded significantly fewer xenomas compared to reference fish exposed to an intraperitoneal injection of spores. In a separate trial, the influence of time, after mussels filter spores for 4 hours, on xenoma development in trout was investigated. While 75% of fish became infected after 4 hours of exposure (similar to previous trials), 10% became infected after 7 days, which indicates that blue mussels may act as a reservoir for infectious *L. salmonae* spores for at least 7 days. A final study was devised to determine whether the

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[1]McConnachie, S.H., Guselle, N.J. & Speare, D.J. (2013). Retention of viable microsporidial (*Loma salmonae*) spores within the blue mussel (*Mytilus edulis*): Use of an experimental laboratory model probing pathogen transfer within a multi-trophic aquaculture setting. *Aquaculture 376(1)*, 1-5.

addition of mussels to *L. salmonae* contaminated water provides bioremediation. The percentage of trout that became infected was similar in fish exposed to contaminated water versus those exposed to contaminated water filtered by mussels. Interestingly, trout exposed to water filtered by mussels, developed higher mean xenoma counts which suggests that viable spores are expelled via mussel feces and pseudofeces, which can then be consumed by trout. Results from these studies have shown that blue mussels, when exposed to a suspension of *L. salmonae* spores during a period of feeding, are very efficient at extracting spores from the water column. The spores remained viable in the mussel, and these spores retained infective potential to salmonids. Therefore, blue mussels have the potential to act as a reservoir for *L. salmonae* when grown in proximity to susceptible salmonid species.

2.2 Introduction

Integrated multi-trophic aquaculture (IMTA) combines the culture of organic extractive species (*e.g.*, bivalves, seaweeds, and benthic invertebrates) with traditional fin-fish culture. The cultivation of species from different trophic levels with complementary ecosystem functions can potentially reduce environmental impacts related to extensive monoculture practices (*e.g.*, nutrient loading; Chopin et al., 2013). However the addition of cultured species in the proximity to fin-fish pens may increase the complexity of disease dynamics within these systems. Specifically, bivalves may add complexity as they often serve as a reservoir for pathogens. For example, *Toxoplasma gondii* remains infective within *Mytilus galloprovincialis* mussels up to 21 days post-exposure (Arkush et al., 2003). Similarly, *Cryptosporidium parvum* oocysts remain infective within *M. galloprovincialis* for up to 14 days post infection (Tamburrini &

Pozio, 1999). Alternatively, within fish farms that are shedding high numbers of infectious pathogens, bivalves have the potential to act as a biological filter (*i.e.*, pathogen sink) to control pathogens shedding off of fin-fish net pens (Chopin et al., 2013).

In some promising reports for fish pathogens, bivalves such as blue mussels (*Mytilus edulis*) and sea scallops can filter and ingest sea lice copepodids (*Lepeophtheirus salmonis;* Molloy et al., 2011; Bartsch et al., 2013). Blue mussels have also been shown to filter and deactivate *Renibacterium salmoniarum* (infectious agent of bacterial kidney disease; Paclibre et al., 1994) and infectious salmon anemia virus (Skår & Mortensen 2007; Molloy et al., 2014). However, blue mussels have been shown to concentrate *Vibrio anguillarum* and infectious pancreatic necrosis virus in high numbers in their feces (Pietrack et al., 2012; Molloy et al., 2013). Fish pathogens with an environmentally stable life stage (*e.g.*, a spore) may become problematic as they would likely remain stable following passage through filtering bivalves. One such environmentally resistant pathogen is *Loma salmonae*, the causative agent of Microsporidial Gill Disease of Salmon (MGDS). *L. salmonae* is an intracellular pathogen which targets cells within the gills, and cause the development of xenomas where spores develop and proliferate. Once xenomas rupture in the gills, the inflammatory response causes a chronic branchitis and subsequent respiratory distress (Speare & Lovy, 2012). MGDS is a leading cause of mortality in farmed Pacific salmonids in Canada, especially for Chinook salmon (*Oncorhynchus tshawytscha*), the most susceptible salmonid (Constantine, 1999). MGDS cannot be reliably treated or prevented, and the environmentally resistant spores are difficult to eliminate within a farm system. One proposed mode of protection against

MGDS is through the anticipated biofiltrative action of bivalves within IMTA operations. Blue mussels can filter particles ranging from 4.0 – 100.0 μm (Reid et al., 2010). Since *L. salmonae* spores range from ~ 2-6 μm (Bruno et al., 1995) they should be filtered by the mussels. Therefore, it would be beneficial to determine how mussels interact with *L. salmonae* spores and how that would translate to disease transfer to farmed salmon species.

A series of laboratory *in vivo* studies were devised to probe the transfer and fate of microsporidial spores of the salmon pathogen *Loma salmonae* to the blue mussel (*Mytilus edulis*). An *in vivo* rainbow trout (*Oncorhynchus mykiss*) branchial xenoma expression model was used to detect the presence of viable microsporidial spores within aquarium-held blue mussels under various conditions. The primary objective was to compare infection rates in rainbow trout following ingestion of mussel tissue following 4 hours of filtration. Trout were used as a model species since Chinook salmon experience severe clinical symptoms and heavy mortality when exposed to *L. salmonae* (See Speare et al., 1998 regarding using rainbow trout as a model for MGDS). The second objective was to determine how long viable spores remained within filtering mussels across an environmentally relevant temperature range (11-21°C). Thirdly, a study examined whether mussels effectively filter *L. salmonae* spores from the water column, reducing the incidence of disease to fish exposed to previously filtered water.

2.3 Materials and methods

2.3.1 Experimental design and study animals

All experiments were completed in accordance with the guidelines of the Canadian Council on Animal Care (CCAC, 2005). The juvenile rainbow trout (~25 g) used for this study were acquired from a commercial hatchery (Ocean Trout Farms Inc., Brookvale, PE, Canada) with no previous history of *L. salmonae*. Positive and negative control groups were utilized in each experiment to verify our disease model. Rainbow trout are resistant to *L. salmonae* after one exposure and thus a positive disease outcome would verify that fish were previously unexposed to the pathogen (Rodriguez-Tovar et al., 2006). Prior to experimentation, the trout where held in a 900 L circular fibreglass tank in a quarantine room. Fish were exposed to a 12 hour photoperiod in 11± 0.3˚C, well-aerated water, with a flow-rate of 2 L minutes^{-1}. Fish were fed 2% of their body weight three times per week (Corey Aquafeeds, Fredericton, NB, Canada). When used for experimentation, fish were transferred to a separate room at least one week in advance. At this time, fish were fed dexamethasone (dex; Dexasone, Valeant, Montreal, QC, Canada) coated feed at a dose of 300 mg kg^{-1} food three times per week. Dex is an immunosuppressant that allows for approximately 5-fold higher infections in trout exposed to *L. salmonae* compared to trout not exposed to dex (Lovy et al., 2008). Water conditions were kept constant, with water temperature at 15 ± 0.3˚C but fish were randomly allocated into 70 L fibreglass tanks hooked up to a fresh water flow-through system.

Aquaculture-derived blue mussels from Prince Edward Island, Canada were obtained from a local commercial source. *L. salmonae* has not been detected in the

waters used for commercial blue mussel culture. Mussels were placed in 11.3 L totes containing artificial seawater (17 ± 0.3˚C ranged 30-35 parts per thousand; ppt, Instant Ocean®, United Pet Group Inc., Cincinnati, OH, U.S.A.). The mussels were acclimated for 7 days preceding experimentation, and were fed algae paste (Innovative Aquaculture Products Limited, Skerry Bay, Lasqueti Island, BC, Canada) three times per week, with daily water changes.

2.3.2 Preparation of L. salmonae spores

Rainbow trout heavily infected with *L. salmonae* xenomas (*i.e.,* presence of white cysts filled with spores on gills) were euthanized by an overdose of benzocaine (120 mg L^{-1}; 4-Aminobenzoic acid ethyl ester, Sigma-Aldrich, Oakville, ON, Canada). The gills were immediately dissected from the fish, removed from the cartilaginous gill arches, and minced using a razor blade. Gill material was then ground in a glass tissue grinder (Wheaton Science Products; Millville, NJ, U.S.A), re-suspended in equal volume of sterile saline (0.85%, 4˚C) and centrifuged (350 xg) for 10 minutes. The supernatant was poured off and the pellet was pressed through a Cellector (Homogenizer, BellCo Glass, Vineland, NJ, U.S.A) metal screen and then filtered again through Nitex mesh (63 µm, Hoskin Scientific, Burnaby, BC, Canada), rinsed with 0.85% sterile saline and centrifuged (350 xg) for 10 minutes. This procedure was repeated, pouring off the supernatant each time. The surfactant Triton-X 100 (6%; 4-(1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol, Sigma-Aldrich, Oakville, ON, Canada) was added to the pellet and vortexed for 30 seconds. The sample was centrifuged (700 xg) twice with equal volume of 0.85% sterile saline for 15 minutes. The supernatant was

poured off and the pellet was re-suspended in 0.85% sterile saline. A dilution was made, when required, with a small amount of spores (0.1 mL) and placed under a hemocytometer (40X; Fisher Scientific, Markham, ON, Canada) in order to determine an estimation of spore concentration.

2.3.3 Trial 1: Viability of spores following exposure to mussels

The objective of trial 1 was to determine whether mussels could transmit infective *L. salmonae* spores following a period of filtration. Seventy naïve rainbow trout were allocated into 4, 70 L circular tanks (N=20 per tank with one tank of N=10) and treated with dex as described in section 2.3.1. Sixty blue mussels were obtained and acclimated as described above in 3 containers (N=20 per container; A, B and C). After the acclimation period, the mussels in container A received 10 mL of purified spore material (7.32×10^6 spores mL^{-1}) and were allowed to filter the spore material for 4 hours. Mussels in containers B and C did not receive any spore material. After the exposure period all containers of mussels were harvested. Container C was harvested first and the resulting mussel tissue and liquor was not exposed to any *L. salmonae* spores. The mussel tissue from container B was removed and promptly injected with 1.5 mL of 7.32×10^5 100 μL^{-1} *L. salmonae* spores in saline to determine whether the mussels somehow deactivated *L. salmonae* upon contact. Mussel tissue and liquor was also removed from container A. The tissue from each of the mussel treatment groups was fed to 3 of the 4 tanks of rainbow trout (the tanks were referred to as Tank A-C depending on the treatment received). The fourth tank containing 10 fish, Tank D, acted as a positive control to determine whether our spores were viable and able to cause heavy *L. salmonae*

infections. The 10 fish were anaesthetized with benzocaine (60 mg L^{-1}) and injected intraperitoneally (IP) with 100 µL of 7.32 $x10^5$ spores per 100 μL^{-1} saline. Fish were maintained as described in section 2.1 for a 6 week period in order to allow the infection to mature. Beginning at week 4 post-exposure (PE), all fish were anaesthetized with benzocaine (60 mg L^{-1}) and non-lethally screened for the presence or absence of xenomas using a dissecting microscope (Stereo-MSA, Wild Leitz Canada Ltd., Willowdale, ON, Canada). At 6 weeks PE, all fish were euthanized via benzocaine overdose (120 mg L^{-1}). From each euthanized fish, the first left gill arch was dissected free for whole-mount observation and the numbers of xenomas were counted using a light microscope (40X, LM; Olympus BH2). The mean number of xenomas per fish was also enumerated, along with the transfer efficiency of the spores exposed to each fish: Transfer Efficiency = number of xenomas per fish/ number of spores exposed x 100. The present and all proceeding data were analyzed with MINITAB® software, version 16 (State College, PA, U.S.A.). For trial 1, one-way ANOVAs were conducted, and significant differences were reported at the α=0.05 level of probability. Bonferroni pair-wise comparisons were completed to determine where differences existed. Normality and homogeneity of variances were observed and suitable transformations were applied where data did not meet normality assumptions.

2.3.4 Trial 2: Mussel tissue infectivity following 4 hour filtration of L. salmonae contaminated water

The objective of trial 2 was to further investigate the claim that *L. salmonae* spores still remain viable and infective following retention by blue mussels. For trial 2 we replaced the spore-injected mussel treatment group with a negative control group that did

not receive any mussel or spore material. Otherwise the procedure remained similar to trial 1, with the addition of a replicate group. Briefly, 160 rainbow trout were randomly allocated into 8 tanks (N=20 per tank), were treated with dex and maintained as described in section 2.3.1. Rainbow trout from 2 of the tanks (N=40) were anaesthetized with benzocaine (60 mg L^{-1}) and injected IP with 6.47 $x10^5$ spores per 100 μL^{-1} saline. Forty blue mussels were obtained and acclimated in 2 containers (N=20 per container). One container was exposed to 4.5 mL of a 3.24 x 10^6 mL^{-1} concentration of purified spore material for 4 hours. Treated and un-treated mussel material was harvested and fed evenly to 4 of the 8 tanks to previously anesthetized trout (N=40 per treatment group). The remaining 2 tanks of fish (N=40) were anesthetized and then returned to their tanks. Fish were maintained and then observed 4 weeks, post-treatment as described in section 2.3.1. Data collection was consistent with the preliminary trial. Nested-design, random effects models were analyzed for each of the independent variables. A Bonferroni-corrected p-value of 0.02 was used for analysis. Data was pooled and analyzed using one-way ANOVAs to determine were differences existed.

2.3.5 Trial 3: Length of time mussels transmit infection to salmonids following initial filtration

The objective of Trial 3 was to assess how long viable *L. salmonae* spores remain within mussels following a 4 hour exposure, and thus able to transmit infection to rainbow trout. One hundred and twenty rainbow trout were obtained and acclimated as described previously in section 2.3.1 (N=20 fish per 6, 70L tanks). Thirty blue mussels were obtained and acclimated as described in section 2.3.1 in a 70 L tank. Five mussels were removed as a negative control prior to adding 10 mL of a 1.00 x 10^7 mL^{-1} spore

concentration solution. Mussels were allowed to filter the spores for 4 hours. After 4 hours, 5 mussels were removed and the water was changed. The remaining mussels were left in the tank and maintained for up to 1 week. Five mussels were removed from the tank after each 24 hour, 72 hour (3 day), and 168 hour (7 day) interval. The mussels were harvested, and the tissue and liquor from the control group and each of the time points were fed to 5 of the tanks of fish, separated by treatment. The remaining tank of 20 trout were anesthetized and injected IP with 1.00×10^5 spores per 100 μL^{-1} saline. Six weeks PE, all trout were assessed for presence of xenomas as described in section 2.3.1. The number of xenomas was compared among treatment groups using the non-parametric Kruskal-Wallis test for the equality of medians for two or more sample groups. Bonferroni-corrected multiple comparisons were analyzed using pair-wise, Wilcoxon-Mann-Whitney tests (α=0.008). The decrease in the incidence of disease was measured over time, using a logistic regression analysis.

2.3.6 Trial 4: Length of time mussels transmit infection across a temperature range (11-21°C)

The objective of Trial 4 was to assess how long viable *L. salmonae* spores remain within mussels following across an environmentally relevant temperature range. Three hundred and seventy-five rainbow trout were obtained and acclimated as described previously in section 2.3.1 (N=50 fish per 70 L tanks). 150 blue mussels were obtained and acclimated as described in section 2.3.1 in a 70 L tank except for the following changes. The mussels were randomly allocated acclimated in 11.3 L totes containing artificial seawater at three different temperatures (N=50 mussels per tote; 11, 16 and 21 ± 0.3˚C ranged 30-35 ppt; Instant Ocean®, United Pet Group Inc., Cincinnati, OH, U.S.A.).

The mussels were acclimated at each temperature for 10 days preceding experimentation and were fed algae paste. Five mussels were removed as a negative control prior to adding 5 mL of a 3 x 10^7 mL^{-1} spore concentration solution to each group. Mussels were allowed to filter the spores for 4 hours. After 4 hours, 5 mussels were removed and the water was changed. The remaining mussels were left in the tank and maintained for 28 days. Five mussels were removed from the tank after each 24 hour, 3 day, 7 day, 14 day, 21 day, and 28 day interval. The mussels were harvested, and the tissue and liquor from the control group and each of the time points were fed to 15 fish, separated by treatment. The control trout were anesthetized and injected IP with 1.00 x 10^5 spores per 100 μL^{-1} saline. Six weeks PE, all trout were assessed for presence of xenomas as described in section 2.3.3. The number of xenomas was Log10 transformed and compared among treatment groups using a two-way ANOVA. Bonferroni-corrected multiple comparisons were analyzed using pair-wise, Wilcoxon-Mann-Whitney tests (α=0.006). The decrease in the incidence of disease was measured over time, using a logistic regression analysis.

2.3.7 Trial 5: Do mussels bioremediate water that is contaminated with spores?

The objective of Trial 5 was to assess whether water that is spiked with *L. salmonae* spores becomes "less infective" to rainbow trout after mussels have been allowed to filter for 4 hours. Specifically, we wanted to determine whether mussels bioremediate water that is contaminated with spores. In the first portion of this experiment, 20 naïve rainbow trout were added to a 70 L tank, allowed to acclimated and fed dexamethasone as described in section 2.3.1. The tank was spiked with1.00 x 10^7 *L. salmonae* spores. Fish were left in the tank for 6 hours with the water flow off and then turned back on. Twenty

more naïve trout were added to another 70 L tank and acclimated and fed dexamethasone-coated feed as described in section 2.3.1. Twenty blue mussels were placed in an 11.3 L tank and left to filter water with 1.00×10^7 spores for 4 hours and then removed and the water was added to a 70 L tank. Again, fish were left in the tank for 6 hours with the water flow off and then turned back on. Six weeks PE, fish were removed and processed as described previously in section 2.3.3 for the presence of xenomas. The incidence of xenomas after 6 weeks was compared using a T-test and confidence interval analysis for 2 proportions. The number of xenomas within fish that did become infected after 6 weeks was compared using a 2-sample t-test.

2.4 Results

2.4.1 Trial 1: Viability of spores following exposure to mussels

The proportion of trout infected 6 weeks PE is summarized in Table 2.1. No xenomas were observed in the negative control group, as expected, and were thus excluded from analysis. It was determined that *L. salmonae* spores remain infective within blue mussels for at least 4 hours following exposure (Table 2.1). Spores did not deactivate upon contact with mussel tissue and that the naïve mussels did not appear to contain any infective *L. salmonae* spores (Table 2.1). The resulting MGSD infection in salmon exposed to filtered mussels is less severe than those directly exposed (*i.e.*, fewer spores transferred, therefore fewer xenomas formed; Xenoma count: $F=3.5$, $df=2$, $p=0.04$; Transfer efficiency: $F=15.1$, $df=2$, $p<0.001$; Table 2.1).

2.4.2 Trial 2: Mussel tissue infectivity following 4 hour filtration of L. salmonae contaminated water

The proportion of trout infected 6 weeks PE is summarized in Table 2.1. No xenomas were observed in our negative control groups as expected and were thus excluded from analysis. There was no evidence of a tank effect (Nested ANOVA; treatment effect; $F=27.5$, $df=1$, $p=0.03$; tank effect: $F=1.6$, $df=2$, $p=0.2$), and thus tank data was pooled. Mean number of xenomas per first left gill arch differed significantly between the treatment groups, with IP injected fish having higher xenoma counts (one-way ANOVA; $F=65.9$, $df=1$, $p<0.001$; Figure 2.1A). IP injected fish had significantly higher of xenoma counts than those fish that were fed filtered mussel material. Spore transfer efficiency was significantly greater in the IP fish compared to fish fed mussel material, and there was no tank effect present (Nested ANOVA; Treatment effect: $F=30.8$, $df=1$, $p=0.03$; Tank effect: $F=2.8$, $df=2$, $p=0.07$; Figure 2.1B).

2.4.3 Trial 3: Length of time mussels transmit infection to salmonids following initial filtration

The proportion of trout infected 6 weeks PE is summarized in Table 2.1. Mean xenoma values differed significantly among the time points (Kruskal-Wallis Test; $H=26.6$, $p<0.001$). Xenoma values were higher at 4 hours compared with 72 hours and 168 hours (Wilcoxon-Mann-Whitney tests; 72 hours: $W=540$, $p<0.001$; 168 hours: $W=547$, $p<0.001$; Figure 2.2). At each hour increase, there is a 0.98 fold decrease in disease transmission and subsequent infection (Logistic Regression equation, $\text{Log(odds)}= 0.59x-0.22$; $G=20.2$, $df=1$, $p<0.001$; Figure 2.2A).

2.4.4 Trial 4: Length of time mussels transmit infection across a temperature range (11-21°C)

The proportion of trout infected 6 weeks PE is summarized in Table 2.1. Mean xenoma values differed significantly among the time points, but the relationship with temperature was insignificant (two-way ANOVA; overall: $F=14.2$, $df=13$, $p<0.0001$; temperature effect: $F=0.03$, $df=1$, $p=0.9$; time effect: $F=28.8$, $df=6$, $p<0.0001$; temperature*time effect: $F=0.1$, $df=6$, $p=0.9$). Multiple comparisons were made using time as a variable only. Mean xenoma count was highest in fish fed mussels exposed for 4 hours compared to all other time points (Table 2.1). At each hour increase, there is a 0.98 fold decrease in disease transmission and subsequent infection (Logistic Regression equation, $Log(odds)= 0.69x-0.0073$; $G=152.8$, $df=1$, $p<0.001$; Figure 2.2B).

2.4.5 Trial 5: Do mussels bioremediate water that is contaminated with spores?

The proportion of trout infected 6 weeks PE is summarized in Table 2.1. For the first part of Trial 5 there was no difference in xenoma presence or absence between the spiked-treated fish or the filtered-treated fish (Test and CI for 2 Proportions; 95% CI [-0.36, 0.26], $Z=-0.32$, $p=0.7$). Fish who were exposed to water that was filtered by mussels had significantly higher numbers of xenomas compared to fish that were in the spiked tank (Two-sample t-test; $T=-4.01$, $df=7$, $p=0.005$; Figure 2.3).

Table 2.1 Summary results for Trials 1-5 using rainbow trout (*Oncorhynchus mykiss*) and blue mussels (*Mytilus edulis*). Treatment groups were as follows for trials 1 and 2; no exposure (naïve), *Loma salmonae* spores injected intraperitoneally (IP), *L. salmonae* spores injected into receiver blue mussels and subsequently fed to receiver rainbow trout (Injected) and, *L. salmonae* fed to filtering mussels whose tissue was then fed to rainbow trout (Filtered). For trial 3, the time series is indicated by the hour. Treatment groups for trial 4 consisted of no exposure (naïve), *L. salmonae* spores injected intraperitoneally (IP) and, *L. salmonae* fed to filtering mussels whose tissue was then fed to rainbow trout across a 28 day period at 3 different temperatures (11, 16 and 21°C). For trial 5, the treatment groups include water spike by spores (Spiked) and water that was spiked with spores, filtered by mussels and then exposed to naive fish (Filtered).

	Proportion with Xenomas on Gills	Mean Xenoma Count on 1st Left Gill Arch in Infected Fish (±SE)
Trial 1		
Naive Mussels	0/20	0
Injected Mussels	14/14	26±7
Filtered Mussels	19/19	6±1
IP Injected Fish	14/14	30±10
Trial 2		
Naive Fish	0/40	0
Naive Mussels	0/40	0
Filtered Mussels	29/39	10±4
IP Injected Fish	40/40	61±10
Trial 3		
Naive Mussels	0/20	0
4 Hr Filtration	15/20	2±0.3
24 Hr Filtration	9/20	1±0.1
72 Hr Filtration	3/20	1±0
168 Hr Filtration	2/20	1±0
IP Injected Fish	20/20	23±8

Trial 4		
IP Injected Fish		
	24/24	124±23
4 hr filtered mussels fed		
Control	0/5	0
11˚C	15/15	42±7
16˚ C	15/15	42±9
21˚C	15/15	63±17
24 hr filtered mussels fed		
Control	0/5	0
11˚C	12/15	12±3
16˚ C	12/15	6±1
21˚C	15/15	9±2
3d filtered mussels fed		
Control	0/5	0
11˚C	1/15	2±0
16˚ C	3/15	2±0
21˚C	6/15	3±0.5
7d filtered mussels fed		
Control	0/5	0
11˚C	3/16	3±0.7
16˚ C	2/16	2±0
21˚C	1/16	2±0
14d filtered mussels fed		
Control	0/5	0
11˚C	3/16	2±0

16˚ C	15/15	2±0
21˚C	1/15	2±0
21d filtered mussels fed		
Control	0/5	0
11˚C	0/14	0
16˚ C	2/15	2±0
21˚C	1/15	2±0
28d filtered mussels fed		
Control	0/5	0
11˚C	1/15	2±0
16˚ C	0/15	0
21˚C	2/14	3±1
Trial 5		
Spiked Water	9/20	3±1
Mussel Filtered	8/20	13±7

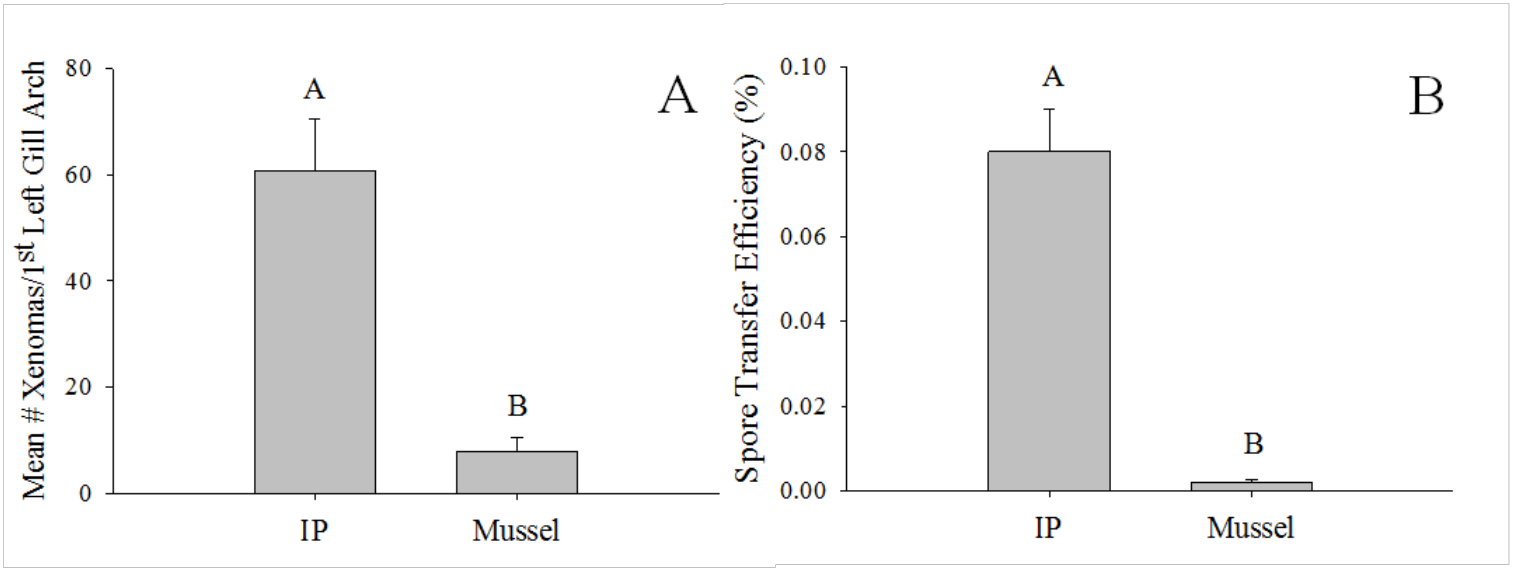

Figure 2.1A-B. Mean number of xenomas per first left gill arch (A), and spore transfer efficiency (B). Full models for each variable were analyzed using a Nested, random effects model. Further analysis was completed with pooled and unpooled data to determine where differences existed (one-way ANOVA). Data for the mean number of xenomas per left gill arch was square-root transformed. Data for the mean xenoma count per fish was log-transformed. Spore transfer efficiency was analyzed as ArcSin(Square Root(Value)). Multiple comparisons were completed using Bonferroni adjusted multiple comparisons and variable letters are reported where differences existed.

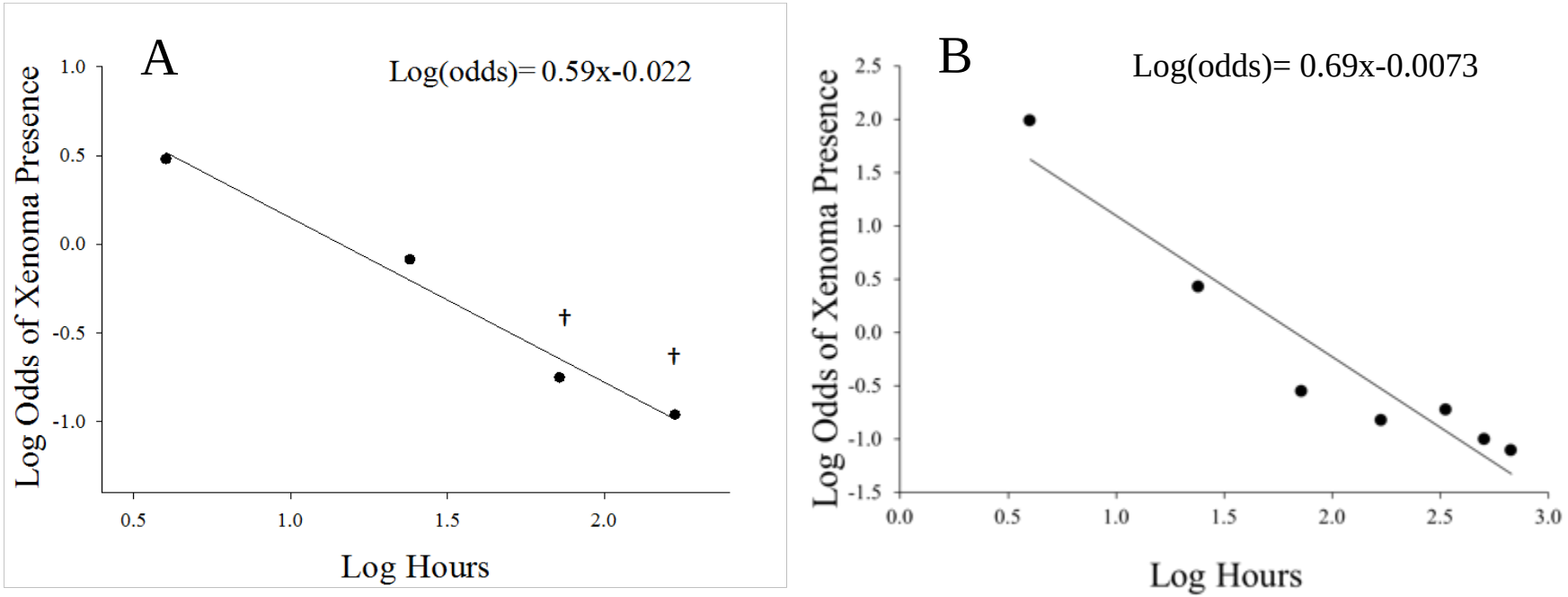

Figure 2.2A-B. Log odds of xenoma presence in rainbow trout (*O. mykiss*) vs. the log hours of filtration for blue mussels (*M. edulis*). **A**: The full model for the binary logistic regression was significant (coef = -0.022, Log odds=0.98, df=1, $p<0.0001$), indicating that xenoma presence decreased over time following a 4 hour filtration period. '†' signifies a significant difference in xenoma counts as compared to the 4 hour period as analyzed using pairwise, Wilcoxon-Mann-Whitney tests for multiple comparisons (Bonferroni-adjusted $\alpha=0.008$) following a Kruskal Wallis test to determine differences in xenoma presence over time ($H=26.6$, df=3, $p<0.001$). **B**: The full model for the binary logistic regression was significant (coef = -0.0073, Log odds=0.98, df=1, $p<0.001$), indicating that xenoma presence decreased over time following a 4 hour filtration period. '†' signifies a significant difference in xenoma counts as compared to the 4 hour period as analyzed using pairwise, Wilcoxon-Mann-Whitney tests for multiple comparisons (Bonferroni-adjusted $\alpha=0.002$) following a Kruskal Wallis test to determine differences in xenoma presence over time ($F=30.8$, df=1, $p=0.03$; Tank effect: $F=2.8$, df=2, $p=0.07$; Figure 1B).

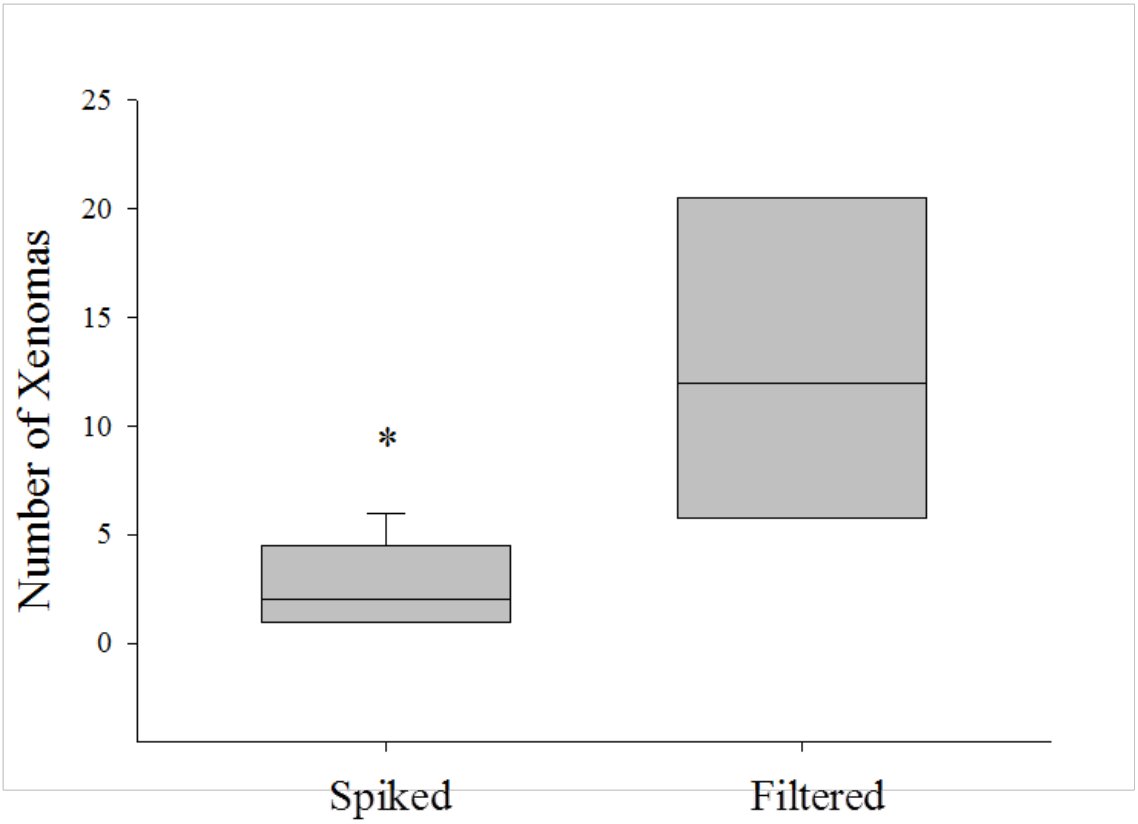

Figure 2.3. Box plot depicting the relationship in mean xenoma counts for rainbow trout (*O. mykiss*) that were exposed to water that was spiked with a known concentration of spores, versus a group of trout that were exposed to water that had previously been filtered with blue mussels (*M. edulis*), for 4 hours. Data was analyzed using a 2-sample t-test to compare the means (T=-4.01, df=7, p=0.005).

2.5 Discussion

The present suite of laboratory experiments elucidates the interaction of *L. salmonae* spores with blue mussels, and how that transfers to subsequent infection in rainbow trout. Initial studies determined that *L. salmonae* spores remained infective to rainbow trout after filtration and passage through blue mussels for at least 7 days. Further study revealed that infective spores remain within blue mussels for at least 28 days, and this period of retained infectivity was not affected by the water temperature at which the mussels were housed (range tested 11-21° C). Infection incidence decreased significantly over time, suggesting the possibility that *L. salmonae* can be captured by mussels, and then flushed out through depuration over a set amount of time. However, it also appears the blue mussels capture and expel *L. salmonae* spores through their feces and pseudofeces, which can then be consumed by fish. In conclusion, the results from all executed experiments reveal that blue mussels act as a reservoir for MGDS.

In comparison to studies that reveal that mussels deactivate infectious particles (*e.g.,* Sea Lice copepodids, Molloy et al., 2011; Infectious Salmon Anaemia [ISA], Skår & Mortensen, 2007, Molloy et al., 2014; Bacterial Kidney Disease [BKD], Paclibare et al., 1994), it does not appear that *L. salmonae* spores are degraded in any way following ingestion and passage through the digestive system. Although the direct test for infectivity proved useful, it could be interesting to conduct a real-time PCR to determine how long *L. salmonae* material remains within the tissue (Skär & Mortensen, 2007). It would also be ideal to determine the passage of *L. salmonae* spores through the digestive tract of the blue mussel and identify where spores persist. Observations can be done by allowing mussels to filter-feed spores and then dissecting each organ, which can be

analyzed using quantitative polymerase chain reaction (qPCR) (*e.g.*, Molloy et al., 2011), microbiological, and histopathological techniques.

There were no differences in spore retention by mussels across the tested temperature range. Feed requirements for weight maintenance of blue mussels increases with increasing temperature, because of higher metabolism at higher temperatures (comparison of 7 and 14°C; see Handå et al., 2013). Therefore, it was theorized that filtration of spores at lower temperatures would be less efficient. However, there were no differences in infectivity across the temperature range, suggesting that mussels would be able to act as a reservoir throughout the year. Water temperatures in the Pacific, where *L. salmonae* susceptible species are farmed in Canada, rarely falls below 5°C or rises above 20°C (DFO temperature data). MGDS infections commonly occur in the later summer and early fall, presumably when water temperatures have increased to ~15°C, which is apparently the optimum temperature for *L. salmonae* infections (Speare & Lovy, 2012). If spores can persist within mussels or in the sediment after the period of shedding during an outbreak, they may remain in proximity and available to fish during the following summer season.

Where *L. salmonae* and MGDS are endemic, it is likely that blue mussels may act as a reservoir in an IMTA scenario. In addition, mussels may act as a reservoir in situations where mussels are biofouling a net-pen, as fish would have direct access to mussels for consumption. Mussels do filter spores and retain them, but they also seem to expel a large number, leaving viable spores in the surrounding environment. Viable spores could accumulate in the sediments surrounding mussel farms, and act as a disease source if that sediment is agitated. Additionally, if mussel fecal matter filled with viable

spores can reach fish by travel through the water column, the fish may ingest the feces, meaning this would be a direct infection source of *L. salmonae*. Temperature does not appear to influence the retention period. Salmonids could obtain direct infection of *L. salmonae* if they happen to consume filtering blue mussels. This phenomenon is not a surprise, given that a recent study by Pietrak and colleagues (2012) determined that blue mussels bioaccumulate and shed viable *Vibrio anguillarum* bacteria into the sediment. Other microbes that are at risk of bioaccumulating surrounding the sediment in mussel farms include: *Toxoplasma gondii* oocysts (Arkush et al., 2003), human-associated *Enterococcus spp.* (Roslev et al., 2009), and *Cryptosporidium parvum* oocysts (Tamburrini & Pozio, 1999). In the case of *L. salmonae* and other fish pathogens, it is suggested that the risk of passage to farmed salmon can be reduced by strategic placement of the mussel portion of integrated farm systems (Pietrak et al., 2012). Further investigation is required to determine how long spores can persist in the sediment surrounding mussel farms and in what fashion they can be re-suspended, and how that affects nearby salmon cages.

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CHAPTER 3.0 *LOMA SALMONAE* VIABILITY FOLLOWING FILTRATION AND EXCRETION BY BLUE MUSSELS (*MYTILUS EDULIS*)

3.1 Abstract

Filtering blue mussels (*Mytilus edulis*) readily extract fish-infecting microsporidian spores (*Loma salmonae*) from the water column. Mussel viscera fed to naïve rainbow trout (*Oncorhynchus mykiss*) using an established *L. salmonae*-rainbow trout disease model determined that spores remain viable within mussels for at least 28 days. However, the fate of spores upon ingestion is unknown. In the present study, feces and pseudofeces from mussels exposed to *L. salmonae* spores were fed to rainbow trout to determine infectivity after excretion. Then, a monoclonal antibody-propidium iodide exclusion test (MAb-PI) specific for *L. salmonae* spores was applied to mussel feces and pseudofeces to determine spore presence and viability. Finally, mussel digestive glands were dissected from mantle tissue and mixed with spores to see if digestive tissue decreased *in vitro* viability. Individual blue mussels were housed in 500 mL of salt water and acclimated for 48 hours prior to experimentation. Spores purified from *L. salmonae* infected rainbow trout were enumerated and exposed at a concentration of 7.5×10^5 spores per mussel (~1,500 spores mL^{-1}). At regular intervals, the feces and pseudofeces were sampled and individually processed. The number of spores per sample was calculated over 7 days. *In vivo* results revealed that feces and pseudofeces from spore-exposed mussels are infective to rainbow trout. Mussels effectively extract spores from the water column, with >99% of spores being removed in experimental housing within 4 hours. The majority of spores were released from with mussel feces within 6 and 24 hours of exposure. Spores were detected in low numbers by 2 days post-exposure (PE) and up to 7 days. Spores could be

extracted from homogenized mussel tissue 7 days PE, but were not detected in the feces or pseudofeces after 7 days. In summary, when mussels are exposed to an inoculation of spores, they are able to ingest the majority of them and excrete them in feces within 24 hours. Membrane integrity of detected spores did not appear to decrease over time, but further study is needed to differentiate between viable and empty spores. Viable spores are likely trapped within mantle and gill tissue, accounting for long-term infectivity of exposed mussels. These studies further demonstrate that filtering bivalves, such as blue mussels have the capacity to act as a reservoir to *L. salmonae* and other environmentally resistant pathogens.

3.2 Introduction

It is widely accepted that bivalve filtration rates vary based on algal concentration (Winter, 1973), and mussels are able to modulate particle retention efficiency based on concentration changes (Riisgard et al., 2003; Strohmeier et al., 2012). Particle capture and ingestion by filtering bivalves is not just a simple mechanical sieving process by the latero-frontal ciliary tracts (Strohmeier et al., 2012). Mussels can preferentially capture particles within mixed suspensions, especially in relation to avoiding sediment ingestion and seasonal availability of particular resources (Newell & Shumway, 1993; Shumway et al., 1997; Strohmeier et al., 2012). Variable capture efficiency is likely controlled by adjusting the interfilamentary spaces of the stenidium (Cranford & Gordon, 1992), and by adjusting the movement and coordination of the latero-frontal cilia (Dral, 1967). Mussels do alter filtration rates based on food availability (Schulte, 1975) in that they respond to food stimulus by increasing their rates of ventilation, filtration and oxygen consumption (Thompson & Bayne, 1972). However, mussels filter water at greater rates in low

concentrations (1500 to 3200 mL hour^{-1}; Schulte, 1975) and filter at very low rates in high concentrations (250 mL hour^{-1}; Schulte, 1975). Below the saturation threshold, and above the critical concentration for filtration, suspended food particles are transferred directly to the mussel stomach and digestive gland (Riisgard et al., 2011).

Suspension feeding bivalves must also differentiate between edible and non-edible seston (Sierszen & Frost, 1992; Beninger & Venoit, 1999). Non-edible or rejected seston results in the formation of pre-ingestive rejecta called pseudofeces, which is the final rejection step during particle processing (Owen, 1974). As an example, blue mussels (*Mytilus edulis*) have a homorhabdic filibranch gill type. This mantle rejection tract contains long cilia (Beninger & Venoit, 1999) and a high density of acid mucopolysaccharide-secreting mucocytes to allow anchoring of the rejected particles to be transported against the direction of water flow (Beninger & St-Jean, 1997). The mantle rejection tracts and fecal expulsion tracts are quite separate in blue mussels allowing easy differentiation between feces and pseudofeces (Beninger & Venoit, 1999).

Particulate matter that enters the digestive gland can be rapidly digested. The digestive diverticula in blue mussels consist of a paired gland containing numerous blind-ending tubules embedded in connective tissue and hemal sinuses (Morten, 1983). The epithelium of digestive tubules is composed of two mature cell types; the columnar acidophilic lysosome-rich digestive cell; and the pyramidal basophilic secretory cell (Owen, 1972; Morten, 1983). Food material is degraded extracellularly in the stomach and then digested intracellularly within the lysosomal system of digestive cells in digestive tubules (Morten, 1983). It would seem likely that pathogens within the water column internalized by digestive cells would be deactivated by such lysozymes.

In fact, blue mussel digestive gland extracts are rapidly lethal to *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease in salmonids (Paclibare et al., 1994). The results from this study indicate that bacteria do not live long within mussel digestive tracts. Similarly, blue mussel digestive gland homogenates appear to rapidly deactivate infectious salmon anemia virus (ISAV) within 24 hours, although fecal pellets were not observed for virus (Skår & Mortensen, 2007; Molloy et al., 2014). It is possible that infectious particles can be released in fecal pellets. Molloy and colleagues (2014) also theorize that viable virus particles can bypass the digestive tract through passage in pseudofeces. In fact, mussels do have the ability to act as a reservoir for many pathogens. Environmentally resistant pathogens, such as *Vibrio anguillarum* and infectious pancreatic necrosis virus (IPNV) bioaccumulate within mussel digestive tissues and are shed into the environment still viable (Pietrak et al., 2012; Molloy et al., 2013). Thus, an important vector mechanism in filtering bivalves is the bioconcentration of pathogens that are then deposited into the environment were is it accessible to susceptible organisms (*e.g.,* fish).

Relevant to the present study, *Loma salmonae* is the causative agent of microsporidial gill disease of salmon (MGDS). The spore is the infectious particle and is utilized as a model for environmentally resistant pathogens within integrated multitrophic aquaculture systems (IMTA; McConnachie et al., 2013). Filtering blue mussels readily extract spores from the water column. Mussel viscera fed to naïve rainbow trout (*Oncorhynchus mykiss*) using an established *L. salmonae*-rainbow trout disease model determined that spores remain viable within mussels for at least 28 days (McConnachie et al., 2013; Chapter 2). However, it is unknown whether the majority of ingested spores are retained within

mussel viscera or are expelled into the environment. If spores are released into the environment they have two pathways: they are ingested and pass through the digestive tract or they are collected in the mantle rejection tract and expelled within pseudofeces. It is unclear whether the components of either tract have the ability to deactivate *L. salmonae* spores.

It is interesting to note that the bioremediation experiment performed in Chapter 2 revealed that the water containing mussel feces after filtering *L. salmonae* spores resulted in heavier infections than directly inoculated water. It is inferred that the spores were concentrated into the mussel feces, which fish directly consumed. As such, this present chapter describes a series of experiments that were designed to determine the fate and viability of spores after filtration by blue mussels. First, feces and pseudofeces from mussels exposed to *L. salmonae* spores were fed to rainbow trout to determine infectivity. Then, a monoclonal antibody-propidium iodide exclusion test (MAb-PI) specific for *L. salmonae* spores was applied to mussel feces and pseudofeces to determine spore presence and viability. Finally, mussel digestive glands were dissected from mantle tissue and exposed to spores to see if digestive tissue decreased *in vitro* viability.

3.3 Materials and Methods

3.3.1 Study animals

Juvenile rainbow trout (~25 g) were acquired from a commercial hatchery (Ocean Trout Farms Inc., Brookvale, PE, Canada) with no previous history of *L. salmonae*. Prior to experimentation, the trout were held and maintained in quarantine, as described in Chapter 2. When used for experimentation, fish were transferred to a separate room at least one week in advance. At this time, fish were fed dexamethasone (dex; Dexasone, Valeant, Montreal, QC, Canada) coated feed at a dose of 300 mg kg^{-1} food three times per week. Dex is an immunosuppressant that allows for approximately 5-fold higher infections in trout exposed to *L. salmonae* compared to trout not exposed to dex (Lovy et al., 2008). Water conditions were kept constant, with water temperature at 15 ± 0.3˚C but fish were randomly allocated into 70 L fibreglass tanks supplied by a well water flow-through system.

Commercial-sized, aquaculture-derived blue mussels from Prince Edward Island, Canada were obtained from a local commercial source. *L. salmonae* has not been detected in the waters used for commercial blue mussel culture. Mussels were placed in 11.3 L totes containing artificial seawater (17 ± 0.3˚C ranged 30-35 ppt; Instant Ocean®, United Pet Group Inc., Cincinnati, OH, U.S.A.). The mussels were allowed to acclimate for 7 days preceding experimentation and were fed algae paste (Innovative Aquaculture Products Limited, Skerry Bay, Lasqueti Island, BC, Canada) three times per week, with daily water changes.

3.3.2 Preparation of L. salmonae spores

Donor rainbow trout heavily infected with *L. salmonae* xenomas (*i.e.*, presence of white cysts filled with spores on gills) were euthanized by an overdose of benzocaine (120 mg L^{-1}; 4-Aminobenzoic acid ethyl ester, Sigma-Aldrich, Oakville, ON, Canada). The gills were immediately dissected from the fish, removed from the cartilaginous gill arches, and minced using a razor blade. Gill material was then ground in a glass tissue grinder (Wheaton Science Products; Millville, NJ, U.S.A), re-suspended in equal volume of sterile saline (0.85%, 4˚C) and centrifuged (350 xg) for 10 minutes. The supernatant was poured off and the pellet was pressed through a Cellector (Homogenizer, BellCo Glass, Vineland, NJ, U.S.A) metal screen and then filtered again through Nitex mesh (63 µm, Hoskin Scientific, Burnaby, BC, Canada), rinsed with 0.85% sterile saline and centrifuged (350 xg) for 10 minutes. This procedure was repeated, pouring off the supernatant each time. The surfactant Triton-X 100 (6%; 4-(1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol, Sigma-Aldrich, Oakville, ON, Canada) was added to the pellet to separate spores from cellular debris, and vortexed for 30 seconds. The sample was centrifuged twice with equal volume of 0.85% sterile saline (700 xg) for 15 minutes. The supernatant was poured off and the pellet was re-suspended in 0.85% sterile saline. A dilution was made, when required, with a small amount of spores (0.1 mL) and placed under a hemocytometer (40X; Fisher Scientific, Markham, ON, Canada) in order to determine an estimation of spore concentration. Spores were stored at 4ºC and used within 2 weeks of processing.

3.3.3 In vivo infectivity of blue mussel feces and pseudofeces following exposure to L. salmonae spores

Materials from Trial 4, Chapter 2 were utilized to determine the infectivity of the fecal material and water from spore-exposed mussels. Briefly, mussels acclimated to 3 different temperatures (*N=50* mussels per tote; 11, 16 and 21 ± 0.3˚C ranged 30-35 ppt; Instant Ocean®, United Pet Group Inc., Cincinnati, OH, U.S.A.) were exposed to 4.5 x 10^8 spores for 4 hours. During the time period mussels were actively filtering water and producing feces and pseudofeces (Figure 3.1). The water from each tote was carefully poured through 63 µm Nitex mesh into 1 L containers. The material on the mesh was re-suspended into sterile saline and centrifuged (700 xg) to produce a concentrated pellet. The filtrate was also centrifuged (700 xg) in order to produce a concentrated pellet. The pellets were aspirated through a 25-gauge needle in order to separate any spores from particulate matter to ensure homogenous samples. The filtrate and excrement samples were separated by temperature. Meanwhile, gelatin power (Oxoid®) was added to boiling water (2 g in 35 mL per sample) and allowed to cool to 30˚C. Then, a drop of red food colouring was added to the gelatin mixture to increase palatability to rainbow trout. As the temperature of the gelatin approached 25˚C, the filtrate and excrement pellets were mixed into the gelatin samples and refrigerated at 4˚C until solid. Once solidified, each sample was macerated into bite-sized pieces. The 6 samples were fed to fish in 6 separate tanks (N=10 fish per treatment group). In another tank, negative control fish were fed processed gelatin (N=5), and in yet another tank, positive control fish were fed gelatin processed with purified spores that had not been exposed to mussels (N=10). Fish were observed closely during the feeding procedures to ensure all fish were consuming gelatin

pieces. Fish were moved into a common tank 2 days after the feeding procedure, and maintained as described in section 3.3.1.

At 6 weeks post-exposure (PE), all fish were euthanized via benzocaine overdose (120 mg L^{-1}). From each euthanized fish, the first left gill arch was dissected free for whole-mount observation and the numbers of xenomas were counted using a light microscope (40X, LM; Olympus BH2). The presence or absence of xenomas was documented, as was mean number of xenomas per fish. The present and all proceeding data were analyzed with MINITAB® software, version 16 (State College, PA, U.S.A.). Xenoma outcome was compared with temperature and sample type (filtrate or excrement) as explanatory variables in a 2-way ANOVA.

3.3.4 Fate and in vitro viability analysis of Loma salmonae spores following filtration and excretion by blue mussels

Mussel pseudofeces and feces were easy to distinguish and sample (Figure 3.1). As such, an experiment was designed to determine the fate and viability of *L. salmonae* spores after filtration by mussels (Figure 3.2). In November-December 2012, mussels (N=11) were individually housed in 800 mL containers with 500 mL of saltwater. Aeration was provided, and mussels were acclimated for 48 hours and observed closely to ensure they were actively filtering water. After 48 hours, ~7.5 x 10^5 spores (~1.5 x 10^4 spores mL^{-1}) suspended in sterile saline were added to the containers. A subsample of spores added to each container was taken to determine *in vitro* viability. One hour PE, any feces and pseudofeces produced were removed from the container using a sterile, 3 mL syringe and emptied into a 15 mL tube. Samples were also taken at 2 and 4 hours PE. The water mussels were housed in was changed 4 hours PE and kept to calculate how

many spores remained. The mussels and the containers were washed and returned to filter. Further feces and pseudofeces samples were taken 6 hours PE, and then every 24 hours PE up until spores were no longer detected in fecal samples (Figure 3.2). Preliminary experiments revealed spores were no longer detected 7 days PE and thus mussels were sampled daily up until 7 days PE.

Samples were aspirated using a 25-guage needle to separate spores from particulate matter. Samples were then filtered through 20 µm Nitex mesh, centrifuged (350 xg), and re-suspended in 100 µl of a monoclonal antibody (MAb) specific to components of *L. salmonae* spores (produced in-house by J. Sheppard; Speare *et al.* 1998) and incubated for 30 minutes in microcentrifuge tubes. After incubation, the material in the microcentrifuge tubes were washed with sterile saline 2x and incubated with 25 µL of 1:200 fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG for 15 minutes. Samples were washed 2x and exposed to 25 µl of 1 mg mL^{-1} propidium iodide fluorescent stain for nucleic acids (PI; 3,8-Diamino-5-[3-(diethylmethylammonio)propyl]-6-phenylphenanthridinium diiodide; Sigma-Aldrich). Cell membrane integrity excludes PI from staining viable cells. Samples were washed 2x and re-suspended in 50 µl of sterile saline. Samples were observed under a fluorescent microscope and counted using a hemocytometer. Spore numbers and viability (proportion of PI+/- spores) were estimated for each sample and compared using t-tests and ANOVAs. Samples of fresh, purified spores were observed each day as a positive control. Samples from non-exposed mussels were taken at each time point as a negative. Samples of fresh, purified spores were processed without the MAb and with the FITC as a negative control for the MAb + spores.

3.3.5 Homogenization of mussels following spore filtration

As mentioned above, spores were difficult to detect in mussel fecal samples by 7 days PE. Since exposed mussels fed to naïve rainbow trout yield infection for up to 28 days PE, it is was theorized that spores are present in mussels in tissues other than digestive tissues (*e.g.*, trapped within gill filaments). A subsample of mussels (N=5) utilized in section 3.3.4 were collected 7 days PE after their final sampling. The mussels were scrubbed and rinsed with water. The mussel was removed from its shell and homogenized using a glass tissue grinder (Wheaton Science Products; Millville, NJ, U.S.A), re-suspended in equal volume of sterile saline (0.85%, 4˚C) and centrifuged (350 xg) for 10 minutes. The supernatant was poured off and the pellet was pressed through a Cellector (Homogenizer, BellCo Glass, Vineland, NJ, U.S.A) metal screen and then filtered again through Nitex mesh (63 µm and then 20 µm, Hoskin Scientific, Burnaby, BC, Canada), rinsed with 0.85% sterile saline and centrifuged (350 xg) for 10 minutes. The pellet was re-suspended in sterile water and left over night (~12 hours) to allow cells to lyse. The next day the sample was washed in water and centrifuged (350 xg) and aspirated with a 25-gauge needle and placed on an equal volume of Percoll®. The mixture was centrifuged for 30 minutes (700 xg). The supernatant containing fats and cellular material was removed and the pellet was washed in sterile water. The sample was observed as a wet mount and processed using the MAb-PI protocol describes in section 3.3.4. The presence and viability of spores was analyzed.

3.3.6 In vitro spore viability following exposure to blue mussel digestive gland tissue

In order to determine whether mussels have the ability to digest *L. salmonae* spores, the digestive gland was dissected from 10 mussels an homogenized as described above (see Figure 3.3; Paclibare et al., 1994). The mantle tissue from the 10 mussels was also homogenized. The homogenates were pooled and centrifuged (1000 xg) for 30 minutes to yield a clarified supernatant that was then filter sterilized (0.45 µm pore size filter). 3.5×10^7 spores were added to each of the samples, and along with a control sample in sterile saline were incubated at 18˚C. Samples were vortexed 2x per day, and a subsample was taken every 24 hours to determine *in vitro* viability over time.

3.4 Results

3.4.1 In vivo Infectivity of blue mussel feces and pseudofeces following exposure to L. salmonae spores

6 weeks PE, fish fed feces and pseudofeces from exposed mussels had easily detectable xenomas on their gills using a light microscope (Figure 3.4). All positive control fish had xenomas. Negative controls remained free of infection. The initial experiment included a comparison of fish exposed to mussel feces and filtrate at 3 different temperatures. Infection status and xenoma count did not differ among temperatures and thus data was pooled and tabulated as a comparison between spore-exposed feces and filtrate (2-way ANOVA: F-stat =2.0, p = 0.2, df = 2). A comparison of xenoma count between fish exposed to filtrate and feces was not significant (Wilcoxon rank sum test: W=372, p=0.06, df=1). (Table 3.1)

3.4.2 Fate, and in vitro viability analysis of Loma salmonae spores following filtration and excretion by blue mussels

Samples from non-exposed mussels did not fluoresce green after FITC application (Figure 3.5A). Spores were difficult to detect using light and phase-microscopy, but when observable appeared as ovoid, phase-bright spores ~ 5µm in length (Figure 3.5B). Spores were easily detected using fluorescent microscopy. Spores appeared as bright, apple-green structures (Figure 3.5C) and were confirmed by observing the structure with the fluorescence reduced (*i.e.,* appeared as in Figure 3.5B). Non-viability was also easily determined by the presence of a bright red circular structure within the confines of an apple-green fluorescing spore (Figure 3.5D).

Filtrate analysis of mussels housed individually within 500 mL of saltwater revealed that mussels filtered >99% of spores within 4 hours (remaining filtrate was 0.6±0.08% of initial inoculum of ~7.5 x 10^6). Approximately 50% (±6, range = 22-80%, N=11) of the initial inoculum of spores was recovered from the feces and pseudofeces in the 7 day period PE. A greater percentage of spores was detected in the feces (40±5%) compared to the pseudofeces (9±3%)(Arcsin[(sqrt)data] T-test : t-stat = -5.0, $p < 0.001$, df = 19, N=11; Figure 3.6).

When observed over 7 days, the majority of spores were released within 24 hours PE (Figure 3.7). Of spores detected within pseudofeces, the greatest amount was released within one hour PE (7±3%, Arcsin[(sqrt)data]; 1-way ANOVA, F=10.9, $p<0.001$, df=3). The greatest amount of spores detected within the feces occurred 6 and 24 hours PE (13±3% and 12±3 respectively; Arcsin[(sqrt)data]; 1-way ANOVA, F=18.7, $p<0.001$, df=10). Less than 2% of spores were detected 48 PE at each time point. *In vitro* viability analysis did not reveal any significant change in spore viability over the experimental

period (Arcsin[(sqrt)data]; 1-way ANOVA, F=0.95, p=0.5, df=6). Although spores were not detected after 7 days PE, the feces from exposed mussels tended to contain amorphous material that stained positively with FITC.

3.4.3 Homogenization of mussels following spore filtration

Homogenization and centrifugation on a Percoll® gradient revealed an observable pellet (Figure 3.8). Spores were not easily detectable in the feces 7 days PE, but were readily observable in each tissue homogenate (N=5 mussels). Most observed spores were viable as determined using the MAb-PI assay (Figure 3.8).

3.4.4 In vitro spore viability following exposure to blue mussel digestive gland tissue

The blue mussel digestive gland was easily dissected from the mantle and remaining tissues. The spores exposed to the mussel tissues were 82% viable at the beginning of the experiment. After 24 hours, the MAb-PI test completed on a subsample of spores revealed that *in vitro* viability did not change over the 24 hour period. The control spores were 79% viable, spores from the mantle tissue were 78% viable, and spores exposed to digestive gland tissue was 74% viable. At 48 hours, the control spores were 82% viable, mantle-exposed spores 80% viable and spores from the digestive gland were 79% viable. Significant bacterial growth was observed by 72 hours and the experiment was ceased at this point. (Table 3.2)

Table 3.1 Summary table of xenoma presence and mean (±SE) xenoma count for rainbow trout (*Oncorhynchus mykiss*) exposed to the water and feces from blue mussels (*Mytilus edulis*) 6 weeks PE to *Loma salmonae* spores for 4 hours. Negative and positive control fish were maintained with all treatment groups in a single tank. Two days post-exposure (PE). Negative control fish were fed gelatin, while positive control fish were fed *L. salmonae* spores embedded in gelatin. The feces and water filtrates were also embedded in gelatin prior to being fed to fish.

	Proportion with Xenomas on Gills	Mean Xenoma Count on 1st Left Gill Arch in Infected Fish (±SE)
Negative Control	0/5	0
Positive Control	10/10	50±6
Mussel Filtrate	28/29	17±2
Mussel Feces	20/24	11±2

Table 3.2 Summary data of mean percent viability (SE±) of *L. salmonae* spores within exposed blue mussel (*Mytilus edulis*) feces and pseudofeces. Spores were stained with a monoclonal antibody (MAb), specific to mature *L. salmonae* spores and then fluorescently labelled with anti-mouse IgG fluorescein isothiocyanate (FITC) and propidium iodide (PI). Spore concentrations were estimated using a hemocytometer. Time points not included in the table were not numerous enough to tabulate a reliable figure for viability (*i.e.*, less than 100 spores). Mean viability counts did not differ significantly among groups (Arcsin[(sqrt)data]; 1-way ANOVA, F=0.95, p=0.5, df=6).

	Mean Percent Viability (%±SE)
Inoculum	77±2
1 Hour Pseudofeces	71±4
2 Hour Pseudofeces	77±6
4 Hour Pseudofeces	82±7
4 Hour Feces	72±4
6 Hour Feces	76±2
24 Hour Feces	72±5
48 Hour Feces	82±3

Figure 3.1: Images of blue mussels (*Mytilus edulis*) actively filtering water within laboratory housing. The image depicts the typical appearance of pseudofeces (large arrow) actively being excreted by a mussel close to the exhalant siphon. Pseudofeces typically were light coloured, non-compact, and frequently remained attached to the mussel. Fecal pellets (arrowhead) were typically brown coloured, arranged in coiled casts and fell to the bottom of the housing. The "*" is identifying the byssal threads that mussels produce to anchor themselves on firm substrate

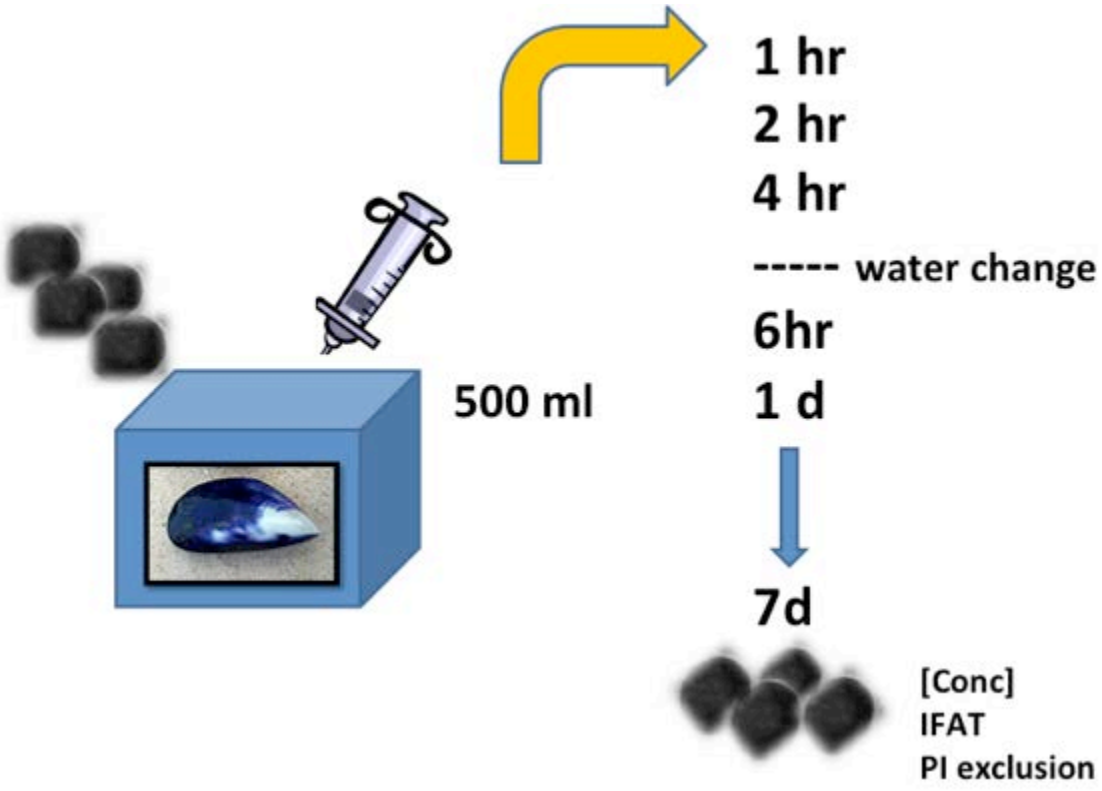

Figure 3.2: Schematic outlining the experimental design for the individual spore exposure trials. Blue mussels (*Mytilus edulis*) were placed in 500 mL of saltwater supplied with oxygen and acclimated for 48 hours. After 48 hours ~750,000 *Loma salmonae* spores were added to the tank. The feces and pseudofeces that each mussel produced was individually samples after 1, 4 and 24 hours. After 4 hours, the water from each tank was removed and set aside for sampling. The tanks and mussels were cleaned and then placed back into more seawater. Further samples were taken at 6 hours and then daily for the following 7 days. For each sample, the concentration of spores was estimated using a monoclonal antibody, propidium iodide (MAb-PI) dye exclusion test.

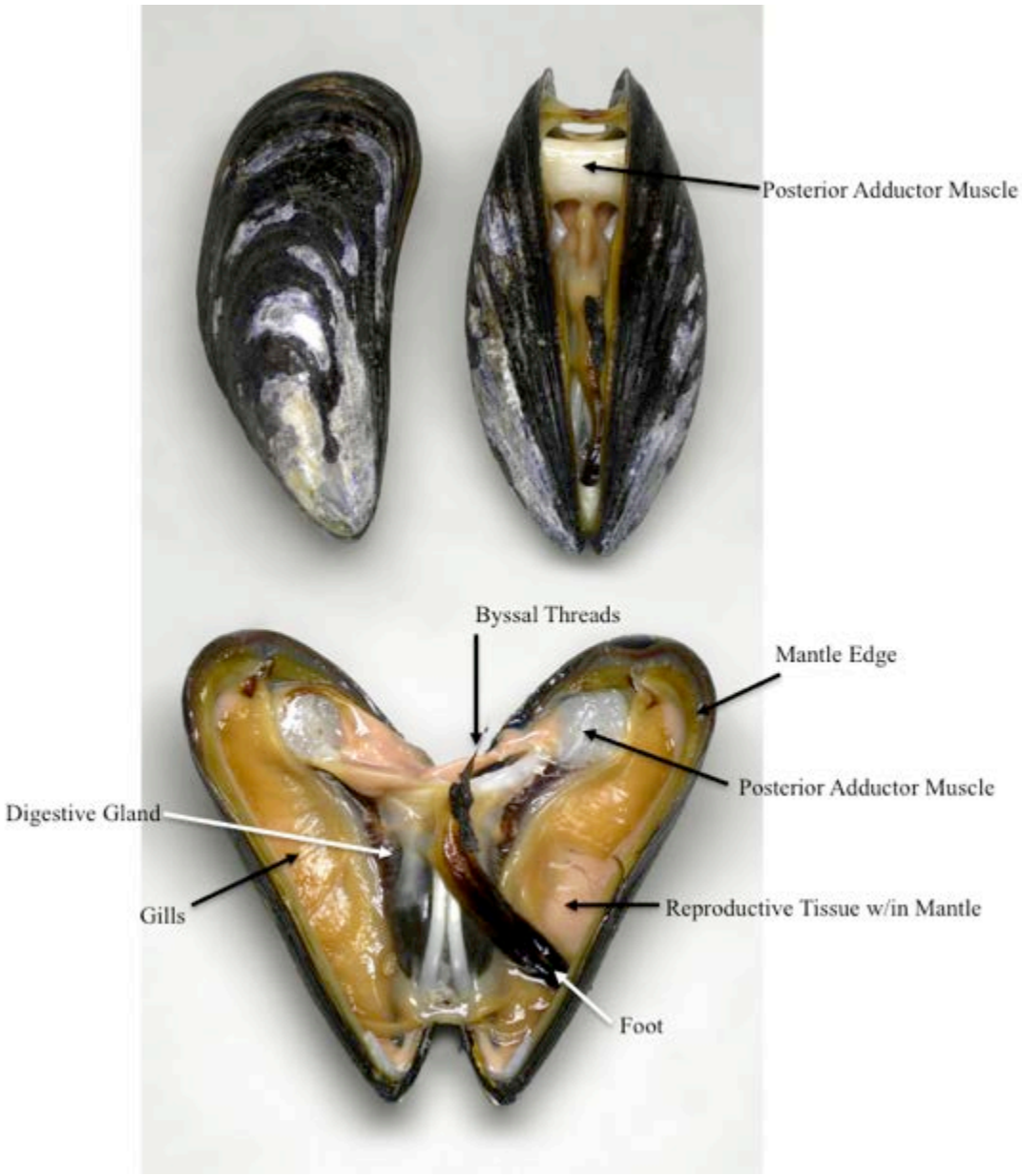

Figure 3.3. Image of the external and internal anatomy of the blue mussel (*Mytilus edulis*). The digestive gland can be easily dissected from the mantle and gill tissue. Image labeled by S. McConnachie. Image by Rainer Zenz (Self-published work by Rainer Zenz) [GFDL (http://www.gnu.org/copyleft/fdl.html), CC-BY-SA-3.0 (http://creativecommons.org/licenses/by-sa/3.0/) or CC BY-SA 2.5-2.0-1.0 (http://creativecommons.org/licenses/by-sa/2.5-2.0-1.0)], via Wikimedia Commons

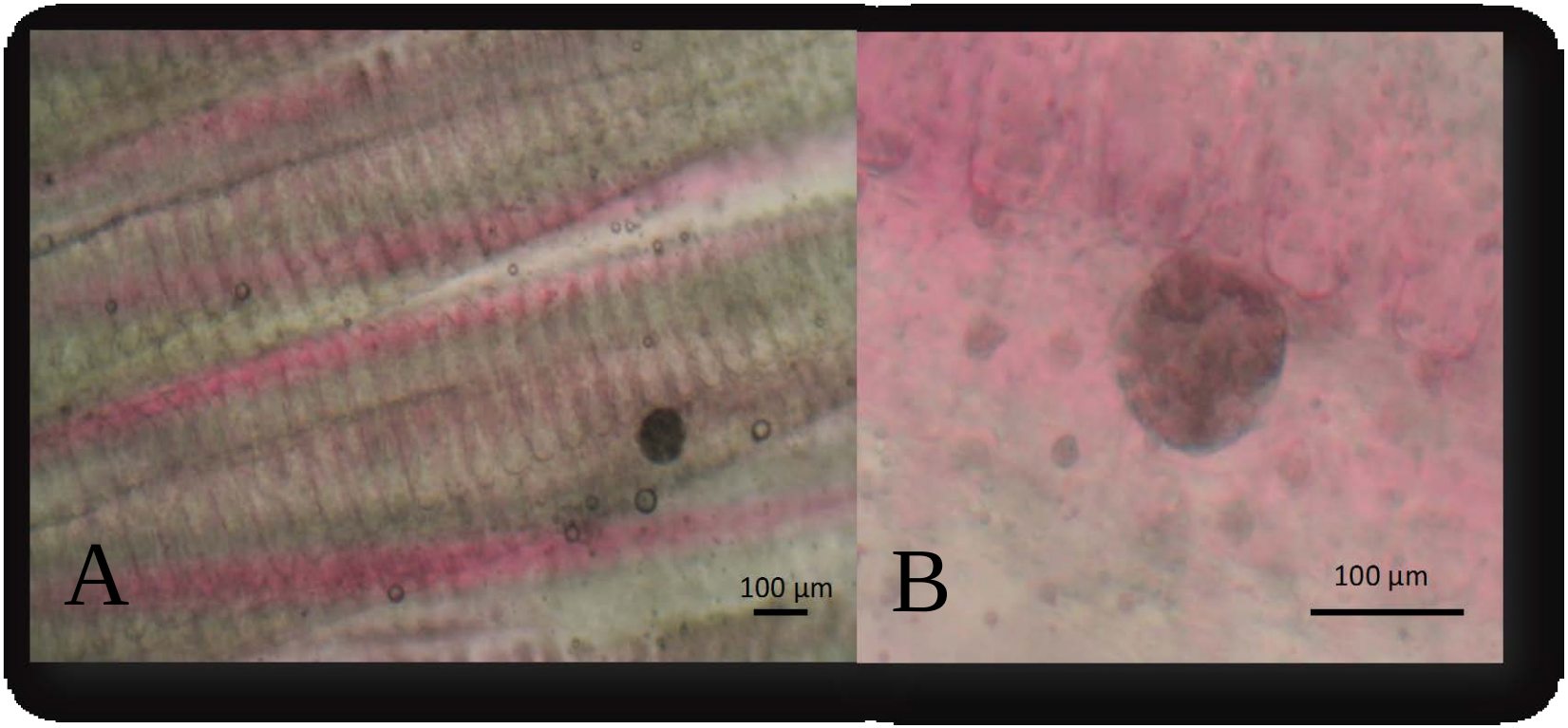

Figure 3.4 (A-B): Example of a typical *Loma salmonae* xenoma seen in the gill tissue of rainbow trout (*Oncorhynchus mykiss*), 6 weeks post-exposure to infective spores. (A) Low magnification light micrograph of a xenoma within the secondary lamellae of a rainbow trout. (B) Higher magnification of a xenoma within rainbow trout gill tissue.

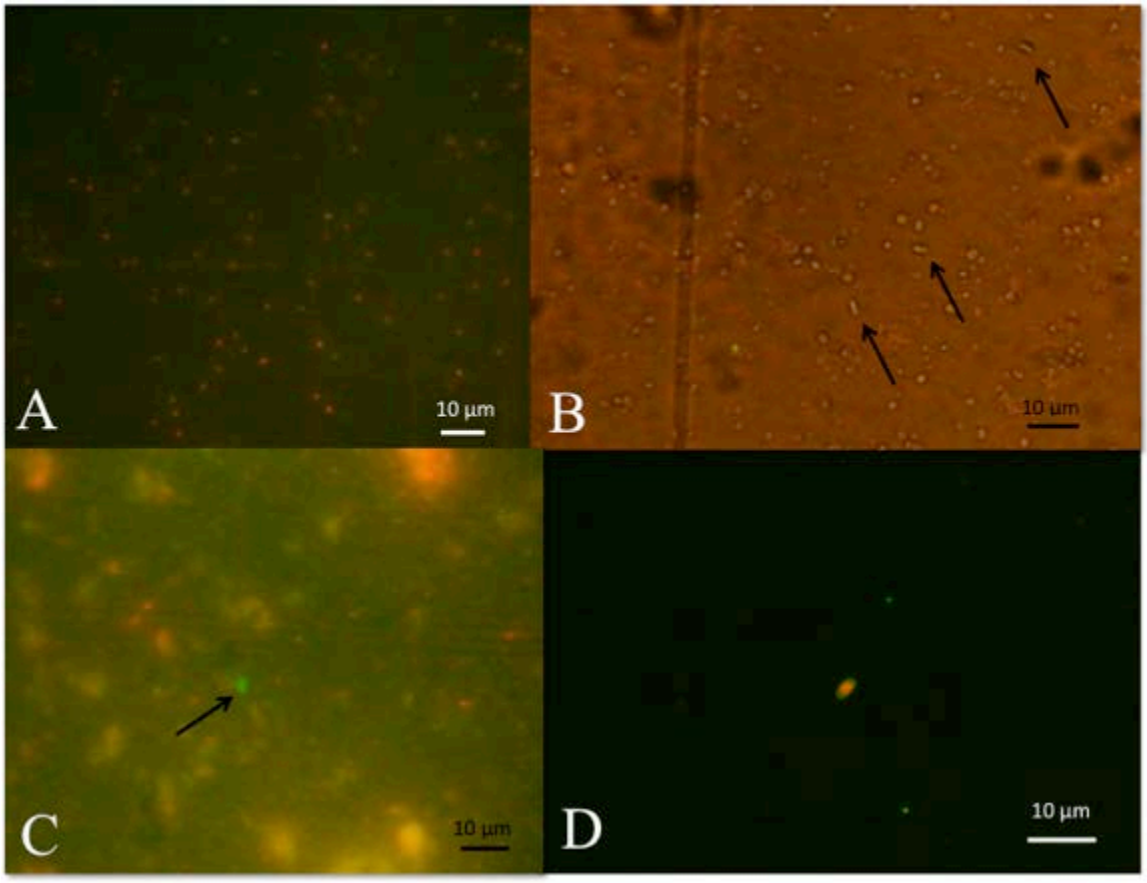

Figure 3.5 (A-D). Fluorescent light micrographs of *Loma salmonae* spores within blue mussel (*Mytilus edulis*) feces 6 hours post-exposure (PE). Spores were stained with a monoclonal antibody (MAb), specific to mature *L. salmonae* spores and then fluorescently labelled with anti-mouse IgG fluorescein isothiocyanate (FITC) and propidium iodide. Spore concentration was estimated using a hemocytometer. (A) Feces collected from a non-exposed mussel. (B) Light micrograph depicting the appearance of spores (arrow) within an exposed mussel. Spores had a characteristic refractile appearance. (C) A fluorescent light micrograph of a *L. salmonae* spore (arrow) and its typical fluorescing appearance. PI has not infiltrated this spore and thus the spore was deemed viable. (D) Appearance of a *L. salmonae* spore (centre) that was deemed non-viable. The spore wall appears to be stained green with the FITC, while PI has infiltrated the spore, making the centre of the spore appear red under fluorescence.

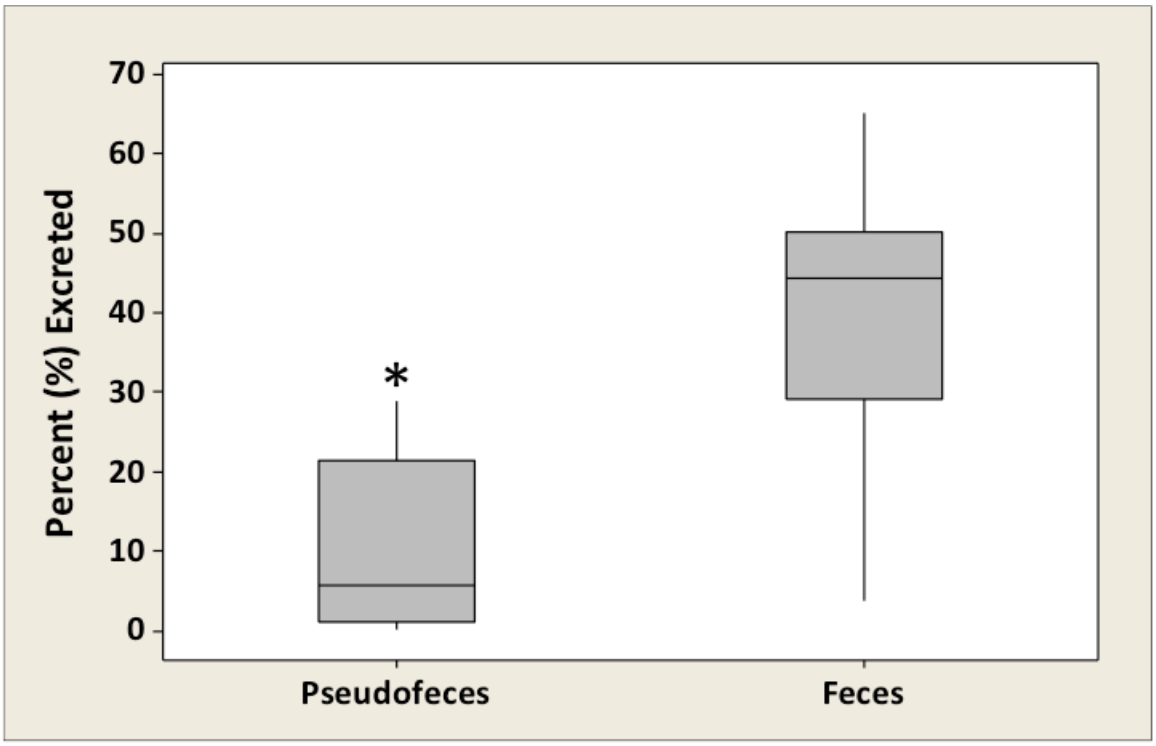

Figure 3.6: Bar graph representing the proportion of *Loma salmonae* spores detected in blue mussel (*Mytilus edulis*) feces using a monoclonal antibody-propidium iodide (MAb-PI) exclusion test. A greater percentage of spores was detected in feces (40 ± 5%) than in pseudofeces (9 ± 3%) (Arcsin[(sqrt)data] T-test : t-stat = -5.0, $p < 0.001$, df = 19, N=11).

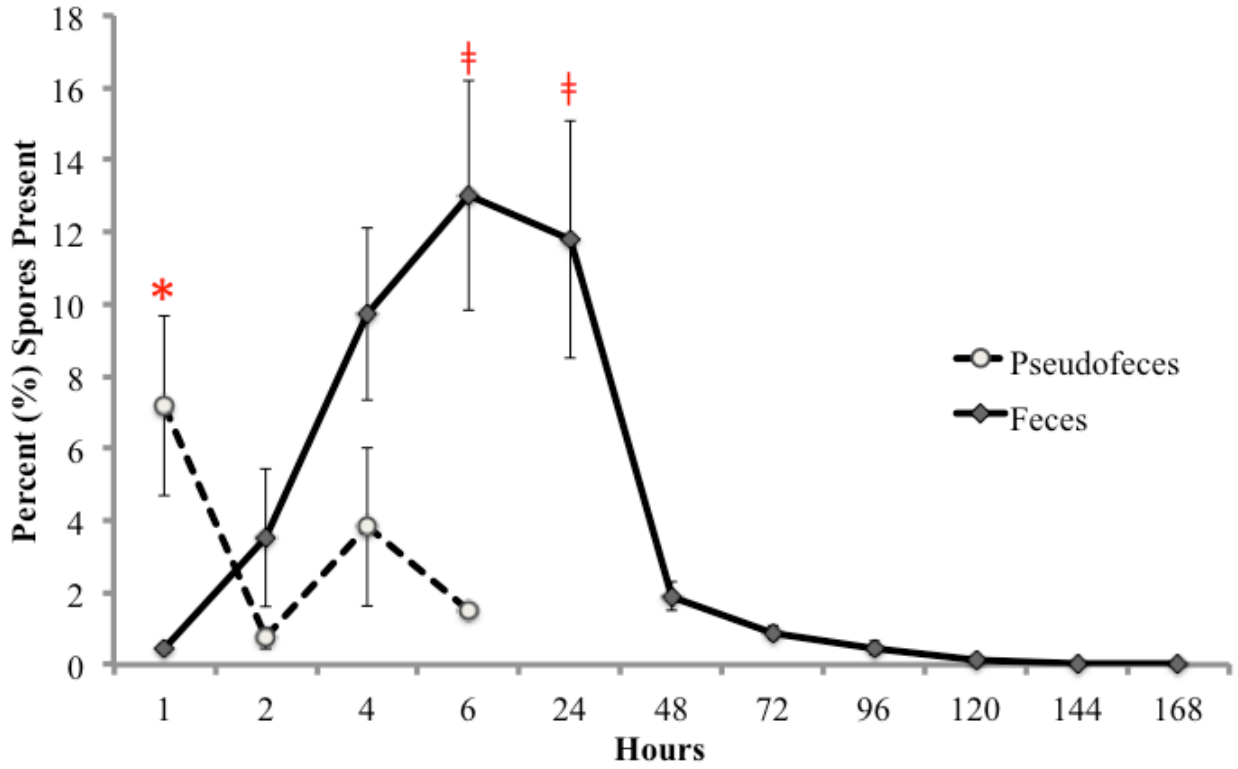

Figure 3.7: Overview of the average (± SE) *L. salmonae* spore count in blue mussel (*Mytilus edulis*) feces and pseudofeces over time (hours). The majority of spores was released in the feces by 24 hours (ǂ) post-exposure (PE). Mussels did not produce pseudofeces after 6 hours PE, and if spores were present, the majority was seen at 1 hour PE (*). The water which the mussels were housed in was changed 4 hours PE. The number of spores left in the water was counted and <1% of the original inoculation remained in the water (750,000 spores). On average, 50% of spores were detected in the feces and pseudofeces in the 7 day period PE (± 6, range: 22-80%, N= 11 mussels). Viability of spores, as detected by a monoclonal antibody-propidium iodide (MAb-PI) dye exclusion test, did not change in the 7 day period PE.

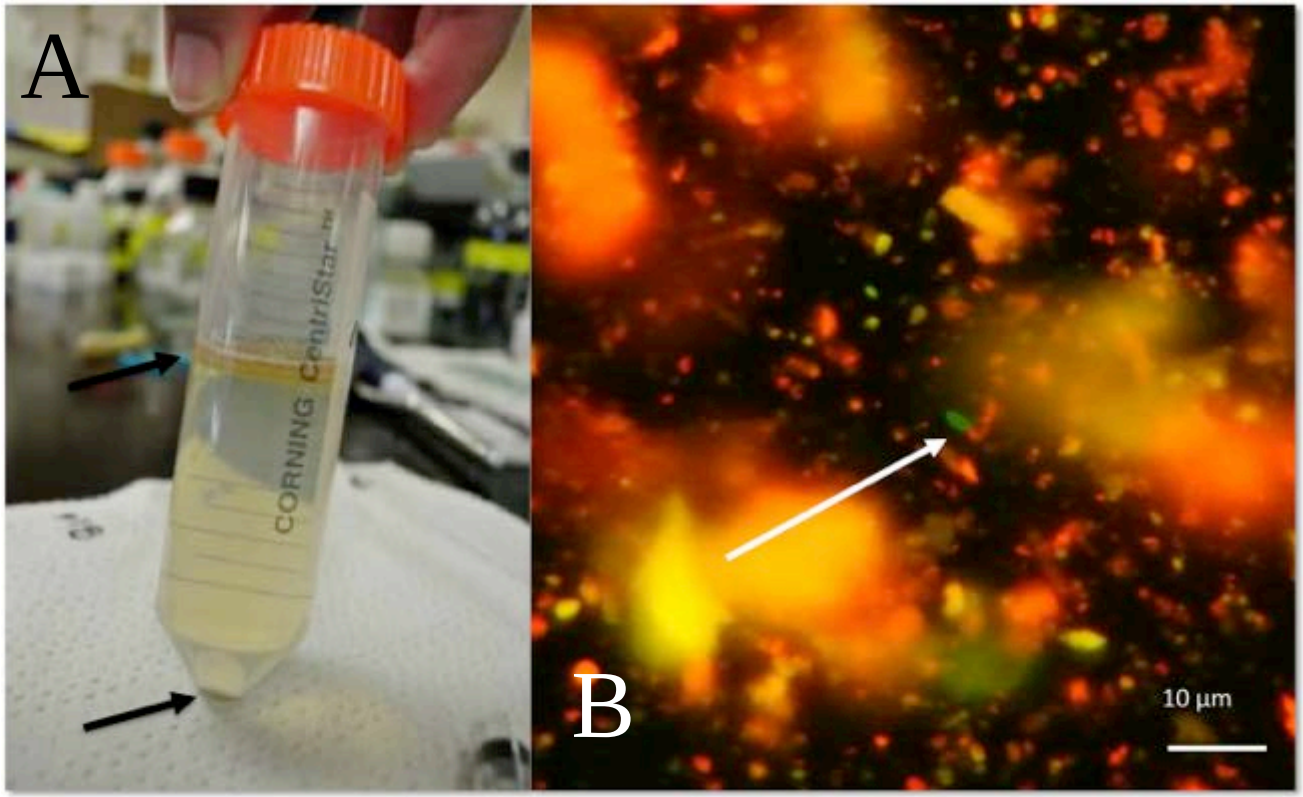

Figure 3.8 (A-B): Image from the experiment involving homogenized blue mussels that had been exposed to *Loma salmonae* spores for 7 days. At this point, spores were not easily detectable in the feces, but spores were still readily observable within homogenized mussel tissue. (A) Appearance of mussel tissue after homogenization and centrifugation with Percoll®. The top arrow is the band of cellular material from the mussel and the bottom arrow points to the material that was stained using a monoclonal antibody-propidium iodide (MAb-PI) exclusion test to identify spores and determine viability. (B) A fluorescent light micrograph of the mussel pellet after staining. The white arrow points to a FITC-positive, empty or viable, *L. salmonae* spore within the homogenate.

3.5 Discussion

IMTA systems can potentially mitigate nutrient loading issues, and may help control important fish pathogens that thrive in monoculture. For example, filtering bivalves ingest and deactivate problematic parasites (*e.g., Lepeophtheirus salmonis*; Bartsch et al., 2013), viruses (*e.g.*, Infectious Salmon Anemia Virus; Molloy et al., 2014), and bacteria (*e.g., Renibacterium salmoninarum*; Paclibre et al., 1994). However, the above pathogens are relatively environmentally fragile with low stability and host diversity. It was theorized that a pathogen with an environmentally resistant life stage, such as *L. salmonae* spores, would remain viable after exposure to filtering bivalves. The studies in Chapter 2 verified that *L. salmonae* spores remain infective following exposure to actively filtering blue mussels for at least 28 days (McConnachie et al., 2013).

Results from Chapter 2 suggested that mussels filter and expel viable *L. salmonae* spores via feces and/or pseudofeces, which could be consumed by fish. Further investigation revealed that >99% of spores were filtered and retained by mussels in a 4 hour period. Spores were primarily expelled into fecal pellets. The majority of spores were released between 6 and 24 hours PE. Spores were detectable in mussel feces for up to 7 days PE. A smaller (but still relevant) proportion of spores were released in pseudofeces up to 6 hours PE. Although spores were not easily detected in feces by 7 days PE, they were readily observed in homogenized mussels suggesting that spores are trapped within gill and mantle tissues where they can remain for an extended time period. Therefore, in an IMTA setting where *L. salmonae* is a concern, mussels have the capacity to act as a disease reservoir.

Data from *in vivo* experiments verify that the excrement from spore-exposed mussels is infective. Results from the *in vitro* viability assessment did not show an increase in spores with damaged membranes (*i.e.,* PI positive) in excrement over time. However, differentiation between viable and empty/germinated spores (*i.e.,* both would be PI negative) was not made so it is unknown what proportion of expelled spores remained infectious. The environment within mussels could induce germination, which warrants further study. Since *L. salmonae* replicates asexually, it only requires a small number of infectious spores to produce a grossly detectable infection. Therefore, even if a small proportion of expelled spores are viable it is still clinically relevant.

Blue mussels effectively removed *L. salmonae* spores from 500 mL of water within 4 hours. *L. salmonae* spores range from 2-6 µm, within the filterable size range for blue mussels (4-100 µm; Reid et al., 2010). Blue mussels have been shown to retain particles larger than 4 µm at 100% efficiency (Møhlenberg & Riisgård, 1978). These results suggest that mussels may act to bioremediate water where many spores are present (*e.g.,* from a net-pen of fish experiencing an active *L. salmonae* infection). However, it appears that mussels can act as a reservoir through the accumulation of viable spores, as seen in Chapter 2. These findings were anticipated as bivalves process large volumes of water (*e.g., M. edulis*, ~1.5 L per hour; Foster-Smith, 1975) and are important screening tools for accumulated human pathogens such as *Cryptosporidium parvum, Giardia duodenalis* and human virulent microsporidians (Fayer et al., 1998; Graczyk et al., 1999; Lucy et al., 2008; Graczyk et al., 2008). Similarly, *Mytilus galloprovincialis* remove *Toxoplasma gondii* oocysts from the water and directly infect sea otters (*Enhydra lutris*

nereis) with high doses of pathogen - resulting in high morbidity and mortality (Arkush et al., 2003).

Spores expelled in mussel feces and pseudofeces were infectious. The association of *L. salmonae* spores within mussel feces may help concentrate spores around culture equipment, rather than dispersing in the environment. This phenomenon is seen in attached *Cryptosporidium* oocysts and *Giardia* cysts (*e.g.*, to leaves and particulate matter; Medema et al., 1998). Sedimentation velocity of attached pathogens has been shown to be much greater than for free infectious particles (Medema et al., 1998). It is unclear how long spores may persist in the environment surrounding mussels and still remain viable. The only studies on *L. salmonae* free-spore viability have been completed in a laboratory setting, but determined spores are viable for at least 100 days in fresh- or sea-water stored at 4°C (Shaw et al., 2000). It is likely that *L. salmonae* spores exist for a similar period in the environment. Environmentally stable pathogens can often persist for long periods of time. For example, *Mycobacterium avium* subsp. *paratuberculosis*, the causative agent for Johne's disease can survive within sediment for at least 48 weeks (Whittington et al., 2005). The human pathogen, *Acanthamoeba* can survive within the environment, even under desiccation, for up to 20 years (Sriram et al., 2008). The concentrated sedimentation of mussel feces-attached *L. salmonae* may help the pathogen persist in susceptible regions.

L. salmonae spores are not rejected by blue mussels, and are readily consumed. Spore presence within pseudofeces suggests that the mussels were size-rejecting spores (*i.e.*, those < 4 µm), or were reaching their saturation point. It would be beneficial to study how the spore concentration within the water column can influence filtration rates,

and thus spore bioaccumulation and deposition. Filtration rates vary based on algal concentration (Winter, 1973), and mussels are able to modulate retention efficiency of particles based on concentration changes (Riisgard et al., 2003; Strohmeier et al., 2012). In fact, the threshold concentration for pseudofeces formation is 1.2×10^4 cells mL^{-1}, and spore concentration in the present study was ~1.5×10^4 spores per mL^{-1} (Riisgard et al., 2011). Below this threshold, and above the critical concentration for filtration, suspended food particles are transferred directly to the mussel stomach (Riisgard et al., 2011). Spore dosage and expected mussel-spore dynamics within an actual IMTA setting requires further examination. In the field, extraction would likely occur at lower concentrations, and spores may have a higher likelihood of being digested. Modeling techniques and spore concentration manipulation could help predict the likelihood of exposure and pathogen accumulation.

This study could not confirm if mussels have the ability to digest *L. salmonae* spores. On average ~50% (range 22-80%) of inoculated spores were retrieved from feces and pseudofeces. It is possible that spores, not captured by the experimental processing techniques, were digested by the mussels. The presence of brightly fluorescing (FITC positive) material in mussel feces 7-14 days PE suggest that a small portion may have been internalized and digested by mussels. Mussels do produce two types of feces, depending on the cell concentration present – glandular and intestinal (van Weel, 1961). When food concentration is high, intestinal feces contain the majority of excreted material. Intestinal feces contain material that has mostly been directly transported into the hindgut, bypassing the digestive diverticula, and is thus less digested (*i.e.*, midgut gland; Owen, 1955). Glandular feces stem from food that has resided in the digestive

diverticula in the presence of digestive cells, and is more thoroughly digested (van Weel, 1961). Undigested algal cells within fecal pellets along with pseudofeces production indicate saturation (Riisgard et al., 2011). It is possible that spores behave similarly to phytoplankton and the spore dose used in this study was too high to determine digestion efficiency. It could be that the dose of spores exposed to mussels surpassed the capacity at which they could be internalized and digested. It would be ideal to alter the spore dose to determine whether the digestive enzymes of the mussels can digest spores. Immunohistochemistry and/or transmission electron microscopy (TEM) could also be applied to mussels that have ingested spores to determine internalization and/or digestion. For example, Robledo and colleagues (1997) utilized conventional and lectin histochemistry in order to characterize the major glycoconjugates of the cell types within mussel digestive glands (*M. galloprovincialis*). These chemicals could be used to probe digestive enzyme association with spores using immunohistochemistry. Similarly, explant cultures of mussel digestive glands have been successful, and it may be possible to develop a protocol that involves the observation of cultured digestive cells with purified spore inoculates (Robledo et al., 1997; Faucet et al., 2004).

Regardless of spore dosage, it is clear that spores can remain viable within mussels for at least 28 days PE. Additionally, spores exposed to mussel digestive tissues did not appear to lose *in vitro* viability, or *in vivo* infectivity – methods that proved highly effective in killing bacterial and viral pathogens (*e.g., R. salmoninarum,* Paclibare et al., 1994; ISAV, Skår & Mortensen, 2007; Molloy et al., 2014). It is important to note that the gill system, protective mantle folds, and pseudofeces rejection tracts are highly mucoid, folded, and ciliated tissues with ample opportunity for particles - pathogenic or

not – to be trapped within them (Beninger & Venoit, 1999; Gomez-Mendikute et al., 2005; Riisgard et al., 2011). Spore retention is likely related to the complexity of such mussel tissues. Further techniques could be employed to investigate this, such as spore localization on mussel tissue histology sections using immunohistochemistry.

Overall, it can be concluded that blue mussels readily extract *L. salmonae* spores from the water column. In high concentrations, viable spores are released into the environment via feces and pseudofeces. It appears that digestive enzymes do not readily digest spores, especially when exposed at high doses. Additionally, viable spores are likely trapped within mantle and gill tissue, accounting for long-term infectivity of exposed mussels. These studies further demonstrate that filtering bivalves have the capacity to act as a disease reservoir to *L. salmonae* and other environmentally resistant pathogens.

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CHAPTER 4.0: USE OF A MONOCLONAL ANTIBODY IN DETERMINING PRESENCE AND VIABILITY OF *LOMA SALMONAE* SPORES UNDER VARIOUS CONDITIONS

4.1 Abstract

Loma salmonae is the causative agent of microsporidial gill disease of salmon (MGDS). An established *L. salmonae*-rainbow trout disease model has revealed much information about the pathogen using a *per os* infection model. The ability of *L. salmonae* spores to cause xenoma development in naïve rainbow trout is the gold standard indicator of spore viability. With the additions of blue mussels (*Mytilus edulis*), and cell culture to the disease model (Chapters 2 and 5), came the need to reliably identify and quantify viable spores within various tissues and excrement. Thus a monoclonal antibody-propidium iodide (MAb-PI) dye exclusion protocol was developed to detect the presence and viability of spores. An experiment was designed to determine if *in vitro* spore viability detected by the MAb-PI protocol, can be used as a proxy for infection outcome *in vivo*. Purified spores were heat inactivated (HI) at 80°C for 1 hour. HI spores and fresh spores were mixed in various ratios: 100% fresh spores (F), 100% HI spores, 75F:25HI, 50F:50HI, and 25F:75HI. The various spore ratios were subsampled and analyzed *in vitro* using the MAb-PI dye exclusion test, and *in vivo* by intraperitoneally injecting spores and observing disease outcome. Results of this experiment suggest a correlation between *in vitro* viability and intensity of infection (*i.e.*, xenoma count). However, further study is needed to differentiate between viable and empty spores. Next, several immunocytochemical applications of the MAb protocol were explored. The MAb-PI protocol is effective at determining the presence of viable spores within blue mussel feces using fluorescence microscopy. A variation of the MAb

protocol involving the immunocytochemical stain, AEC (3-Amino-9-ethylcarbazole) was effective in visualizing spore associations with cultured cells using light microscopy. A MAb-FITC protocol was also developed to observe spore associations with the leucocytes from rainbow trout during *L. salmonae* infection. Lastly, the MAb-PI protocol was applied to a suite of chemical disinfectants that have promise in deactivating *L. salmonae* spores. *In vitro* and *in vivo* data suggest that sodium hypochlorite is the best disinfectant choice for *L. salmonae* spores.

4.2 Introduction

Loma salmonae is the causative agent of microsporidial gill disease of salmonids (MGDS) that is especially problematic within Chinook salmon (*Oncorhynchus tshawytscha*) aquaculture in western Canada. Most information relating to infection with *L. salmonae* has been determined experimentally. A reliable *L. salmonae*-rainbow trout (*O. mykiss*, LS-RT) disease model was first developed in 1998 (Speare et al., 1998). MGDS can be induced by: feeding naïve fish infectious tissue, injecting purified spores intraperitoneally (IP), anal gavage, intramuscular and intravascular injection, co-habitation, and through exposure to contaminated water (Shaw et al., 1998; Becker & Speare, 2004; McConnachie et al., 2013). The LS-RT model was developed when MGDS first became a concern in farmed salmonids. Efforts went towards completing the parasitic life cycle within laboratory fish to learn more about the pathogen and to discover ways to control it. *L. salmonae* can infect *Oncorhynchus* and *Salvelinus* spp. but not Atlantic salmon (*Salmo salar*). Infections are especially problematic in Chinook salmon, as they exhibit the most severe pathology (Ramsay et al., 2002). The sequelae of

infection in rainbow trout is similar, but the pathology is less severe and thus are ideal for use in a laboratory infection model (Kent & Speare, 2005). Generally, infection is induced by exposure to spores *per os* or via IP injection. Fish are maintained in tanks at 15°C for 6 weeks, or until the desired maturity of infection is reached.

Microsporidians are definitively identified using transmission electron microscopy (TEM) and/or PCR techniques, although these methodologies are costly and time consuming (Lom & Dyková, 2005; Didier et al., 1996). Live spores can be non-specifically identified using histochemical techniques such as chitin-staining fluorochromes and nuclear counterstains such as DAPI (4', 6-diamidino-2-phenylindole) (Didier et al., 1996; Monaghan, 2011). Techniques using polyclonal and monoclonal antibodies (MAbs) have been clinically useful for human-infecting microsporidians, such as *Encephalitozoon bieneusi* in stool samples (Accoceberry et al., 1999). Components of the chitin-containing endospore are antigens that cause antibody reactions in mice and rabbits immunized with microsporidia (Accoceberry et al., 1999). The MAb technique as a spore identification assay in human stool samples is highly specific when compared to TEM, PCR, and different staining techniques (Accoceberry et al., 1999). Diagnoses is rapid and can be made in subclinical infections when few spores are present (Accoceberry et al., 1999). MAbs are also useful for the isolation and purification of spores using chromatography or immnomagnetic separation.

The traditional LS-RT model assesses spore viability via the presence of xenomas on trout gill filaments following the six-week incubation period. This model is preferred as it observes the completion of the developmental cycle of the spores *in vivo*, which is necessary to determine spore viability. However, it was important to develop a quick and

simple way to assess the identity and viability of spores in suspension, especially when mixed with mussel excrement. Therefore, the aim of Chapter 4 was to correlate apparent spore viability, via an *in vitro* assay, with spore infectivity *in vivo*. The viability assay developed in the present chapter is a known *L. salmonae* reactive MAb (clone 4H8), combined with a fluorescein isothiocyanate (FITC) and propidium iodide (PI) dye exclusion assay. PI is impermeable to viable cell membranes; it will bind to nucleic acids within compromised cells and emit a red fluorescence that provides an excellent contrast to the green fluorescence of the MAb assay (Amigo et al., 1994; Stober et al., 2001).

First, an experiment was designed to determine if *in vitro* spore viability detected by the MAb-PI, can be used as a proxy for infection outcome *in vivo*. Various spore mixtures containing an increasing ratio of fresh and heat-inactivated spores were subsampled and analyzed *in vitro* using the MAb-PI dye exclusion test, and *in vivo* by IP injection of spores and observing disease outcome. Next, several immunocytochemical applications of the MAb protocol were explored to help clarify spore-cell associations *in vitro*. Lastly, the MAb-PI protocol was applied to a suite of chemical disinfectants that could deactivate *L. salmonae* spores, but had never been explored in depth using *in vitro* validation techniques.

4.3 Materials and Methods

4.3.1. Study animals

The juvenile rainbow trout (~25 g) used for this study were acquired from a commercial hatchery (Ocean Trout Farms Inc., Brookvale, PE, Canada) with no previous history of *L. salmonae*. Prior to experimentation, the trout were held in a 900 L circular fibreglass tank in a quarantine room. Fish were exposed to a set photoperiod in 11± 0.3˚,

well-aerated water, with a flow-rate of 2 L min^{-1}. Fish were fed 2% of their body weight three times per week (Corey Aquafeeds, Fredericton, NB, Canada). When used for experimentation, fish were transferred to a separate room at least one week in advance. Water conditions were kept constant, with water temperature at 15 ± 0.3˚C; fish were randomly allocated into 70 L fibreglass tanks connected to a fresh water flow-through system.

4.3.2. Preparation of L. salmonae spores

Donor rainbow trout heavily infected with *L. salmonae* xenomas (*i.e.,* presence of white cysts filled with spores on gills; Figure 4.1A) were euthanized by an overdose of benzocaine (120 mg L^{-1}; 4-Aminobenzoic acid ethyl ester, Sigma-Aldrich, Oakville, ON, Canada). The gills were immediately dissected and processed using techniques outlined in Chapter 2. Briefly, gill material was ground in a glass tissue grinder, re-suspended in equal volume of sterile saline (0.85%, 4˚C), washed and filtered, rinsed with sterile saline, treated with Triton-X surfactant (4-(1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol), washed and re-suspended in sterile saline. A dilution was made, when required, with a small amount of spores (0.1 mL) and placed on a hemocytometer (40X; Fisher Scientific, Markham, ON, Canada) to estimate the concentration of the spore mixture (Figure 4.1B). Spores were stored at 4ºC and used within 2 weeks of processing.

4.3.3. Preparation of the L. salmonae monoclonal antibody (MAb)

The MAb (clone 4H8) was originally developed by Judy Sheppard (Immunology Technician, Pathology and Microbiology, AVC) as described in Speare et al. (1998). Briefly, *L. salmonae* spores were harvested and purified from the gills of heavily infected

fish. BALB/c mice were immunized with 1 x 10^6 spores suspended in Ribi adjuvant system (Monophosphoryl-lipid A + Trehalose dicorynomycolate adjuvant; Sigma-Aldrich). Three weeks later, the mice received 1 x 10^7 spores without adjuvant. Hybridomas were prepared by standard protocol and were screened for antibody production by immunofluorescence and immunoperoxidase techniques. Several hybridomas were selected and subcloned; the 4H8 clone was used in subsequent protocols (Speare et al., 1998). When additional antibody was required, the 4H8 clone was grown in standard cell culture medium (Chapter 5) and the supernatant, containing *L. salmonae*-specific MAb, was collected.

4.3.4. Validation of the MAb-PI exclusion test in predicting in vivo spore viability

An experiment was designed to determine if *in vitro* spore viability can be used as a proxy for infection outcome *in vivo* for rapid viability assessment. A heat-inactivation (HI) protocol was developed to deactivate spores without the addition of chemicals that may interfere with the staining procedures used (McGowan, 2012). Fresh, purified spores (F) were counted and placed into two 50 mL centrifuge tubes. One tube of F spores were heat inactivated (HI) in an 80° C water bath for 60 minutes. At room temperature (~25° C), F and HI spores were mixed in varying ratios (100%F, 75F:25HI, 50F:50HI, 25F:75HI, 100%HI) and analyzed for *in vitro* viability using three 100 µL subsamples for each treatment group.

Each 100 µL subsample was incubated with 100 µL of the undiluted MAb for 30 minutes in microcentrifuge tubes. After incubation, the material in the microcentrifuge tubes was washed with sterile saline 2x using a microcentrifuge (350 xg) and incubated with 25 µL of 1:200 fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG

for 15 minutes (Sigma-Aldrich). Samples were washed 2x using a microcentrifuge (350 xg) and exposed to 25µL of 1 mg mL^{-1} propidium iodide fluorescent stain for nucleic acids (PI; 3,8-Diamino-5-[3-(diethylmethylammonio)propyl]-6-phenylphenanthridinium diiodide; Sigma-Aldrich). Cell membrane integrity excludes PI from staining viable cells. Samples were washed 2x using a microcentrifuge (350 xg) and re-suspended in 50 µL of sterile saline. Samples were observed under a fluorescent microscope and counted using a hemocytometer in order to determine an estimation of spore concentration. Spore numbers and viability (proportion of PI+/- spores, 500 counted per sample; Figure 4.2) were calculated for each replicate (3) and treatment group (5). Samples of fresh, purified spores were observed at each collection period as positive controls. Samples processed without the MAb and with the FITC were collected as negative controls for the MAb + spores.

For the *in vivo* portion of the experiment, three replicates of 8 fish per treatment group (5) were utilized. The HI and F spore treatment groups were then injected IP into naïve rainbow trout at a dose of ~1.3×10^5 spores per fish. Six negative control fish, IP injected with sterile saline were added to each tank. Six weeks after the IP injections, fish were euthanized and the first left gill arch was dissected and observed as a wet mount. A randomly assigned coding system was used to label treatment groups to ensure that the slides were observed without bias. The proportion of fish infected and the mean (±SE) number of xenomas present on the first left gill arch was tabulated. All values were compared using a 1-way ANOVA. A general linear regression model was performed to determine the relationship of *in vivo* xenoma count in rainbow trout as a function of *L. salmonae in vitro* viability.

4.3.5. Immunocytochemical detection of L. salmonae spores: Detection of spores within blue mussel feces

The experiments in Chapter 3 required the development of a reliable method to identify and quantify the presence and viability of *L. salmonae* in blue mussel feces. Fungi-Fluor® was compared to the MAb-PI exclusion assay. Fungi-Fluor® was chosen because it reliably detects microspordian spores in clinical samples (*e.g.,* feces, sputum, etc.), and does not require any incubation or washing steps that increase the possibility of error. Feces were collected from mussels maintained in the AVC Aquatics Facility as described in Chapter 2. A known concentration of purified spores were added to the fecal samples. An equal volume of spore-feces mixture was added to Fungi-Fluor® stain and directly observed using a fluorescent microscope. Spores concentrations were estimated using a hemocytometer and viability was observed by observation of spore germination under the microscope. Another sample of the fecal-spore mixture was processed using the MAb-PI dye exclusion assay described above. A comparison was made between the concentration and viability of spores observed for each sample.

4.3.6. Immunocytochemical detection of L. salmonae spores: Observations of spore associations with cell cultures

As described in Chapter 5, mosquito cells (*Aedes albopoctus*; AED) are semi-adherent cells that rapidly phagocytize *L. salmonae* spores. They were easy to subsample, as the semi-adherent nature allowed easy removal from culture flasks. Methods for observing spore-cell associations were explored. While observation using transmission electron microscopy (TEM) is the gold standard, it is a lengthy, tedious, and expensive process. An investigation into the rapid and accurate assessment of AED cells and spores included the application of two immunocytochemical protocols. At 10 days PE, cells

were removed from flasks and placed into microcentrifuge tubes. Cells were stained with the *L. salmonae* MAb and then fluorescently labeled with anti-mouse IgG, FITC and propidium iodide (PI) as described above. Cells were observed using fluorescence microscopy. Positive and negative controls consisted of purified spores and cells treated with FITC, but not with the MAb respectively.

Next, an immunocytochemical technique that does not require fluorescence microscopy was explored. Cells and spores, 10 days PE were removed from cell culture flasks, placed on 6 well slides (~20 µL per well) and left to air dry. Samples were blocked (for endogenous peroxidase) and fixed with 5% hydrogen peroxide (H_2O_2) in methanol. Samples were then incubated with 10% normal goat serum in phosphate buffered saline (PBS) for 10 minutes. After washing with PBS, slides were incubated in 1% monoclonal antibody (MAb) in PBS, specific for *L. salmonae* spores for 1-2 hours at room temperature (~25° C). Slides were washed 3x in PBS and incubated with 1/100 horseradish peroxidase (HRP)-conjugate anti-mouse secondary antibody. Finally, slides were incubated with AEC (3-Amino-9-ethylcarbazole) solution (4 mL dionized water, 2 drops acetate buffer, 1 drop AEC solution, 1 drop 3% hydrogen peroxide) for 25 minutes and rinsed with distilled water. Slides were air-dried, covered with mounting medium, coverslipped, and then observed using light microscopy. AEC-positive material should appear to stain intensely red where *L. salmonae* spore-wall antigen is present.

4.3.7. Immunocytochemical detection of L. salmonae spores: Observations of spore associations with rainbow trout leucocytes

Although it is assumed that *L. salmonae* is transported from the gut to the gills of fish via leucocyte circulation, this has never been demonstrated experimentally. An

experiment was designed to investigate the utility of using a MAb-FITC fluorescence protocol in observing spore presence in circulating trout leucocytes. Three rainbow trout from infection maintenance tanks were placed in a separate tank 3 hours after being exposed to *per os* inoculum of spores. 12 hours PE, fish were anesthetized, fin-clipped and blood sampled using a 25 gauge needle and 3 mL syringe. The syringe contained 0.5 mL of heparinized RPMI-1640 medium (Gibco®); 0.5 mL of blood was collected. Samples were stored on ice until they could be processed. The 1:2 dilution of blood was added to another 1 mL of RPMI-1640 and centrifuged (250 xg) to obtain a cloudy supernatant and buffy coat containing leucocytes. The leucocyte/plasma mixture was then placed on an equal volume of Ficoll and centrifuged (700 xg) for 30 minutes to separate red blood cells from leucocytes. After the leucocytes were isolated they were re-suspended in RPMI-1640 and processed for observation using the MAb-FITC protocol described above. Another blood sample was taken at 24 hours PE and then weekly for 4 weeks. At 4 weeks, after subsampling for analysis using the MAb, the cells were lysed in water overnight and placed on a Ficoll gradient to isolate cell material from any *L. salmonae* spores that may be present within cells. The pellet from the Ficoll gradient was washed and processed for observation using the MAb-FITC protocol described above.

4.3.8. Chemical disinfection of L. salmonae spores

Several chemical disinfectants are useful against environmentally resistant spores of microsporidia. However, there are no specific recommendations in place for *L. salmonae* spores. Therefore, an experiment was designed to compare the *in vitro* and *in vivo* viability of spores following the application of various concentrations and types of

disinfectants. Spore preparations were exposed to the following suite of chemicals, all for 30 minutes: sodium hypochlorite (*i.e.*, chlorine, NaClO; 0.5, 5, 100 parts per million; ppm), iodophor (polyvinylpyrrolidone iodine; 100, 300, 400 ppm), hydrogen peroxide (H_2O_2; 1, 10, 30%), 1% Virkon® (Active ingredients: potassium peroxymonosulfate, $KHSO_5$, and sodium chloride, NaCl), 70% ethanol (CH_3CH_2OH), and 1% formalin. *In vitro* viabilities were determined using the MAb-PI protocol. The disinfected spores from the sodium hypochlorite, iodophor, and hydrogen peroxide treatments were then washed and IP injected into naïve rainbow trout (*O. mykiss*). The corresponding *in vivo* infectivity of the processed spores are listed as a proportion of fish infected (*i.e.*, xenomas present) and mean (±SE) number of xenomas present on the first left gill arch.

4.4 Results

4.4.1 Validation of the MAb-PI exclusion test in predicting in vivo spore viability

HI of *L. salmonae* spores at 80°C for one hour was not 100% effective among the 3 subsamples. *In vitro* analysis determined that ~1±0.2% spores remained viable. HI spores were infective, *in vivo*, yielding a 24% infection rate with xenoma counts of 4±2 xenomas per first left gill arch. However, HI spores were significantly less infective than fresh spores, which yielded 84±2% viability *in vitro* and 100% infection *in vivo*, with an average xenoma count of 87±13 per first left gill arch (*in vitro* viability: Arcsin[(sqrt)data]; 1-way ANOVA, $F=135.1$, $p<0.001$, $df=4$; pairwise comparisons using t-tests with pooled SD and Bonferroni-adjusted P-values); (xenoma count: log-transformed; 1-way ANOVA, $F=15.3$, $p<0.0001$, $df=4$; pairwise comparisons using t-tests with pooled SD and Bonferroni-adjusted P-values).

The *in vitro* viability of spores combined with an increasing ratio of HI spores resulted in viabilities that all differed significantly among treatment ratios. *In vivo* infectivity did not yield a similar, significant gradient (Table 4.1). However, xenoma count increased as a function of *in vitro* viability, with strong positive correlation (r^2=0.7623, p<0.0001; Figure 4.3).

4.4.2 Immunocytochemical detection of L. salmonae spores

The MAb-PI protocol was most effective in detecting *L. salmonae* spores within blue mussel feces. FungiFluor® produced vibrant fluorescence that made detecting spores simple within non-contaminated samples. However, within mussel feces, FungiFluor® fluoresced a large amount of material that presumably was of fungal and bacterial origin (*e.g.*, yeasts and *Staphylococcus* spp.; See Figure 4.4A). Although the stain was effective at visualization of spore germination (Figure 4.4B), spores tended to germinate outside of the field of view and thus the protocol was not reliable in determining spore viability. The MAb protocol reliably allowed detection of spores within contaminated mussel samples, and non-viable spores were easy to detect via staining with PI (Figure 4.4C).

The MAb staining protocol also yielded interesting results when applied to isolated rainbow trout leucocytes at various time points after *per os* spore exposure. Although leucocytes from unexposed fish exhibited some background autofluoresence (Figure 4.5), various leucocytes appeared to diffusely stain FITC-positive 24 hours after exposure. Stain intensity increased over the 4 week experimental period (Figures 4.6-4.12). Some leucocytes also appeared to contain granular, amorphous material that stained intensely FITC-positive (Figures 4.9-4.12). Observation of these cells increased

in frequency throughout the experimental period. By 4 weeks PE, some of the granular leucocytes contained what appeared to be whole spores (Figure 4.12). Lysed and processed leucocytes at the 4 week period yielded the presence of spore-like particles within a strong background fluorescence (Figure 4.11).

However, the MAb-PI staining protocol was not efficient in observing *L. salmonae* spores within AED cells after phagocytosis. The images captured (Figures 4.13A-B) were not dissimilar enough to results seen in phase-contrast microscopy (See Chapter 5) to warrant use of the protocol. In comparison, the AEC spore-visualization assay yielded samples with significant contrast that could have utility in observing spore-cell associations in cell culture (Figures 4.14A-E). Spores were easily detected in association with AED cells, and cell cytoplasm often stained weakly AEC-positive, suggestive of spore degradation products that have been incorporated into cells (Figures 4.14A-E).

4.4.3 Chemical disinfection of L. salmonae spores

The *in vitro* viability analysis of spores treated with various disinfectants at differing concentrations appeared to accurately predict *in vivo* viability (Table 4.2). Sodium hypochlorite appeared to be the most effective disinfectant in rendering spores non-viable and non-infective. Low concentrations (0.5ppm) deactivated >99% of spores. Iodophor and H_2O_2 were less effective at deactivating spores in both *in vitro* and *in vivo* trials (Table 4.2). Preliminary, *in vitro* analysis of 1% Virkon, 70% ethanol and 1% formalin suggest that both Virkon and ethanol effectively deactivated spores, while 1% formalin does not (Table 4.2).

Table 4.1 Summary table comparing the *in vitro* viability with *in vivo* infectivity of heat-inactivated (HI) *Loma salmonae* spores. Fresh, purified spores (F) were stained with a monoclonal antibody (MAb), specific to mature *L. salmonae* spores and then fluorescently labelled with anti-mouse IgG fluorescein isthiocyanate (FITC) and propidium iodide (PI) and then observed with fluorescence microscopy. Mean viability (±SE) was determined by the absence of PI infiltration within the spore (3 samples of 500 spores counted). Another group of spores were HI in an 80°C water bath for 1 hour. A subsample of HI spores was set aside and analyzed as described above. Then F and HI spores were mixed in varying ratios (75F:25HI, 50F:50HI, 25F:75HI) and analyzed for *in vitro* viability. Spore treatment groups were then intraperitoneally injected into naïve rainbow trout (*Oncorhynchus mykiss*) at a dose of ~1.3 x 10^5 spores per fish. Fish were euthanized 6 weeks PE and the proportion of fish infected and the mean (±SE) number of xenomas present on the first left gill arch was tabulated. Mean *in vitro* viability differed among the treatment groups as indicated below (Arcsin[(sqrt)data]; 1-way ANOVA, $F=135.1$, $p<0.001$, $df=4$; pairwise comparisons using t-tests with pooled SD and Bonferroni-adjusted P-values). Mean xenoma counts also differed significant among groups (log-transformed; 1-way ANOVA, $F=15.3$, $p<0.0001$, $df=4$; pairwise comparisons using t-tests with pooled SD and Bonferroni-adjusted P-values).

	Mean *in vitro* Viability (%±SE)*	Proportion with Xenomas on Gills	Mean Xenoma Count on 1st Left Gill Arch in Infected Fish (±SE)*
Fresh Spores	84 ± 2^{e}	20/20	87 ± 13^{c}
75F:25HI	66 ± 3^{d}	21/21	56 ± 8^{c}
50F:50HI	48 ± 6^{c}	20/20	76 ± 13^{c}
25F:75HI	25 ± 3^{b}	20/20	45 ± 12^{b}
100HI	1 ± 0.2^{a}	5/21	4 ± 2^{a}
Saline	-	0/18	0

*Superscript letters in the corresponding columns denote significant differences among treatment groups with dissimilar letters.

Table 4.2 Summary of the mean percentage (±SE) of *Loma salmonae* spores killed using various chemical disinfectants at different concentrations. *In vitro* viabilities were determined using a monoclonal antibody-propidium iodide exclusion test (MAb-PI). Disinfected spores were then washed and intraperitoneally injected into naïve rainbow trout (*Oncorhynchus mykiss*). The corresponding *in vivo* infectivity of the processed spores are listed a proportion of fish infected (*i.e.,* xenomas present) and mean (±SE) number of xenomas present on the first left gill arch. Note: Chlorine refers to sodium hypochlorate.

	Mean Percentage of Spores Killed (%±SE)	Proportion with Xenomas on Gills	Mean Xenoma Count on 1st Left Gill Arch in Infected Fish (±SE)
Saline	-	0/30	0
Positive Control	-	30/30	29±4
Chlorine 0.5ppm	99±0.5	0/30	0
Chlorine 5ppm	99±0.2	0/30	0
Chlorine 100ppm	100±0.2	0/30	0
H_2O_2 1%	31±2	9/30	4±0.6
H_2O_2 10%	95±3	8/30	4±0.6
H_2O_2 30%	93±3	8/30	3±0.4
Iodophor 100 ppm	83±4	0/30	0
Iodophor 300 ppm	90±5	6/30	4±0.7
Iodophor 400 ppm	88±4	3/30	3±0.7
Virkon 1%	93±2		
Ethanol 70%	98±3		
Formalin 1%	37±2		

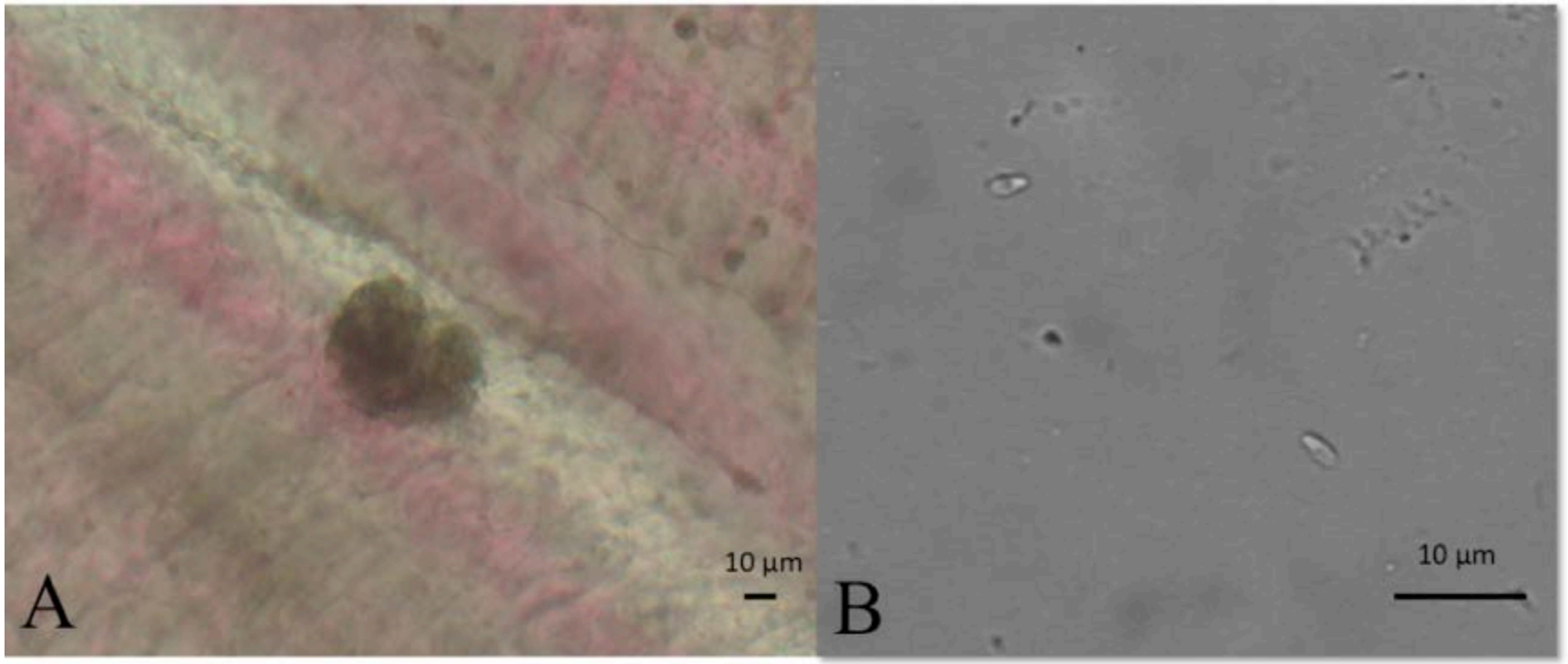

Figure 4.1 (A-B): Light micrographs of *Loma salmonae*. **(A)** Image of a xenoma within the gill tissue of a rainbow trout (*Oncorhynchus mykiss*) 6 weeks post-exposure to *L. salmonae* spores. **(B)** Light micrograph of the typical appearance of spores that have been purified from fish tissue and suspended in saline and placed on a wet mount. Spores are refractile using phase-contrast microscopy. The spores appear opaque at the anterior region and transparent where the large posterior vacuole resides.

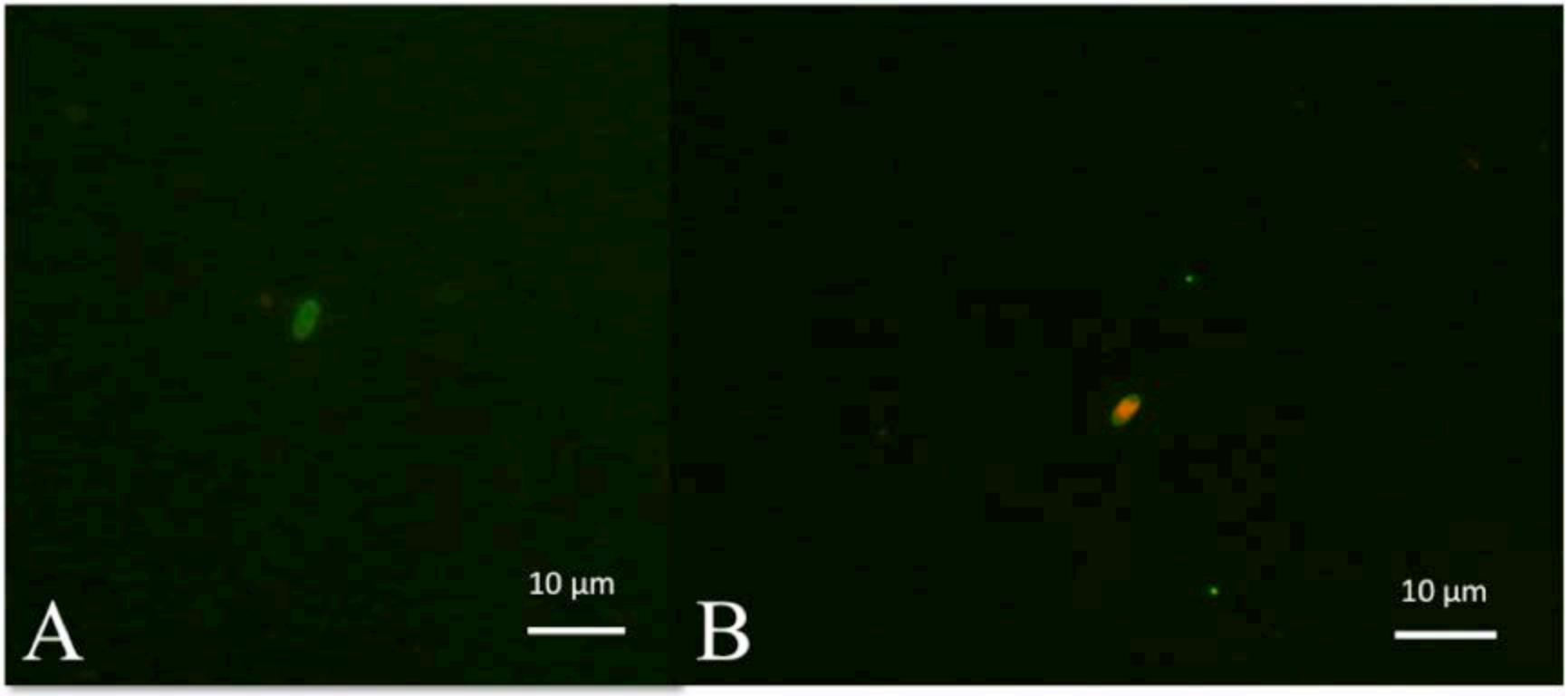

Figure 4.2 (A-B): Fluorescent light micrographs of *Loma salmonae* spores stained with a monoclonal antibody (MAb), specific to mature *L. salmonae* spores and then fluorescently labelled with anti-mouse IgG fluorescein isothiocyanate (FITC) and propidium iodide (PI). The MAb-PI staining procedure allowed for the detection of non-viable spores (*i.e.*, PI enters cells with permeable membranes**). (A)** In the centre is a bright, apple-green, fluorescing spore. **(B)** Appearance of a *L. salmonae* spore (centre) that was deemed non-viable. The spore wall appears to be stained green with the FITC, while PI has infiltrated the spore, making the nucleic acid rich centre of the spore appear red.

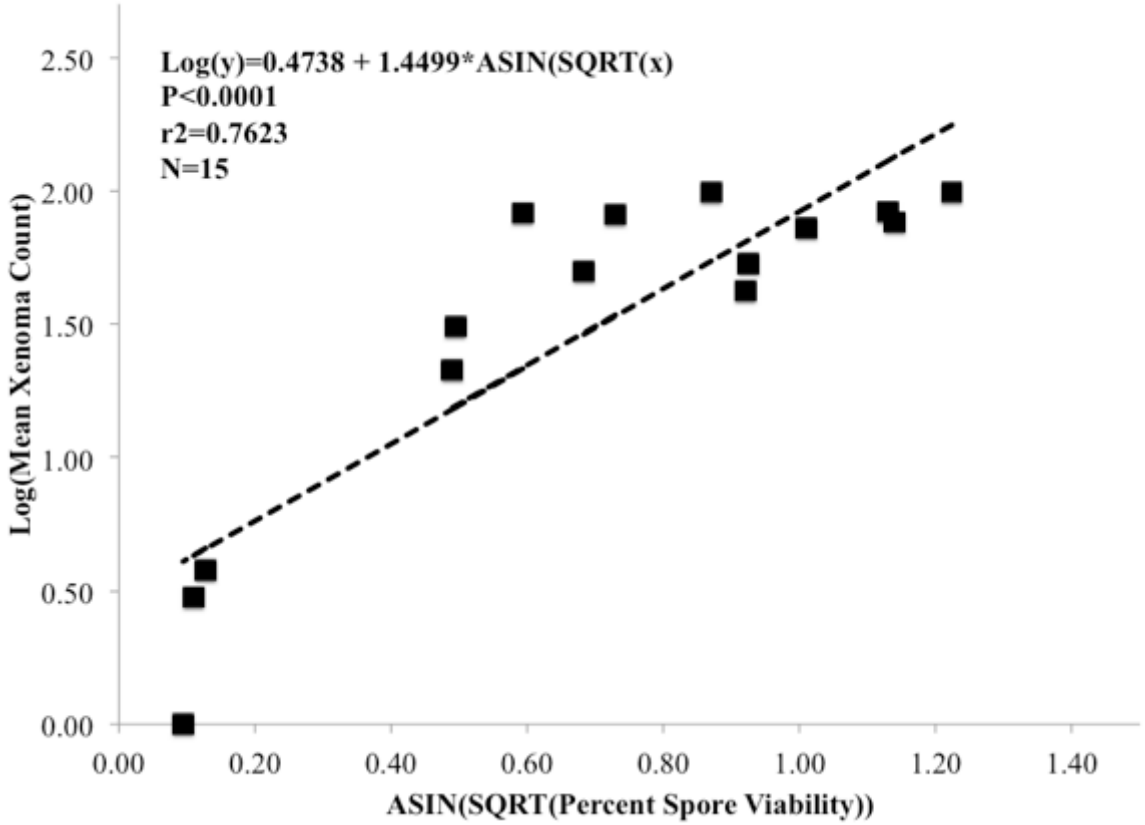

Figure 4.3 Relationship of *in vivo* xenoma count in rainbow trout (*Oncorhynchus mykiss*) as a function of *Loma salmonae in vitro* viability. Xenoma counts increased as percent (%) spore *in vitro* viability increased. Data was transformed for analysis: Spore viability was transformed using ArcSin(Square root) values, and the Log_{10} value for mean xenoma count was utilized.

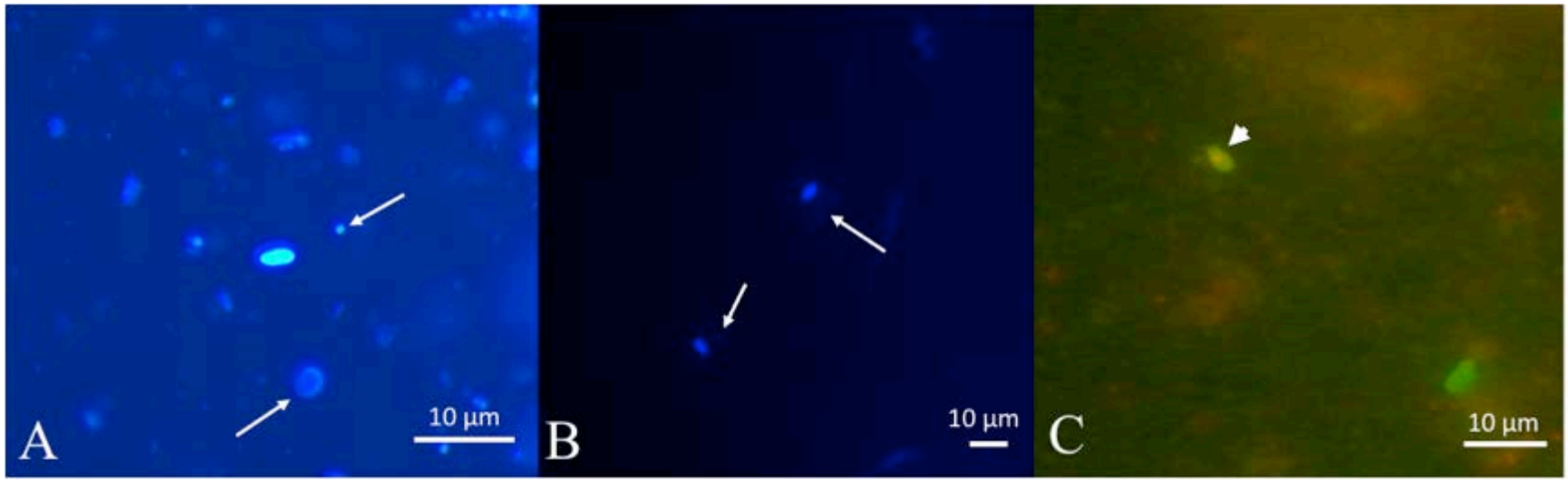

Figure 4.4 (A-C). Fluorescent light micrographs of *Loma salmonae* spores to compare staining techniques. **(A)** Spore stained with Fungi-Fluor®, a dye containing calcifluor white which detects fungal organisms, such as microsporidians. This spore was in a sample of blue mussel (*Mytilus edulis*) feces that had been exposed to *L. salmonae*. Spores did fluoresce brightly, but the stain also picked up unrelated fungal organisms within the fecal samples (arrows). **(B)** Spores stained with Fungi-Fluor® evert their polar tube (light fluorescing material surrounding each spore; arrows) when the fluorescent light source is focused on an ungerminated spore. The action of polar tube eversion can determine spore viability. However, when spores tended to germinate while out of view the metric was not reliable. **(C)** Spores within mussel feces stained with a monoclonal antibody (MAb), specific to mature *L. salmonae* spores and propidium iodide (PI). The MAb-PI staining procedure allowed for the detection of non-viable spores (*i.e.,* PI entered cells with permeable membranes). The spore (arrowhead) with a red centre, indicates PI has entered the spore and is thus non-viable. The bottom spore is fully green and is either empty or viable.

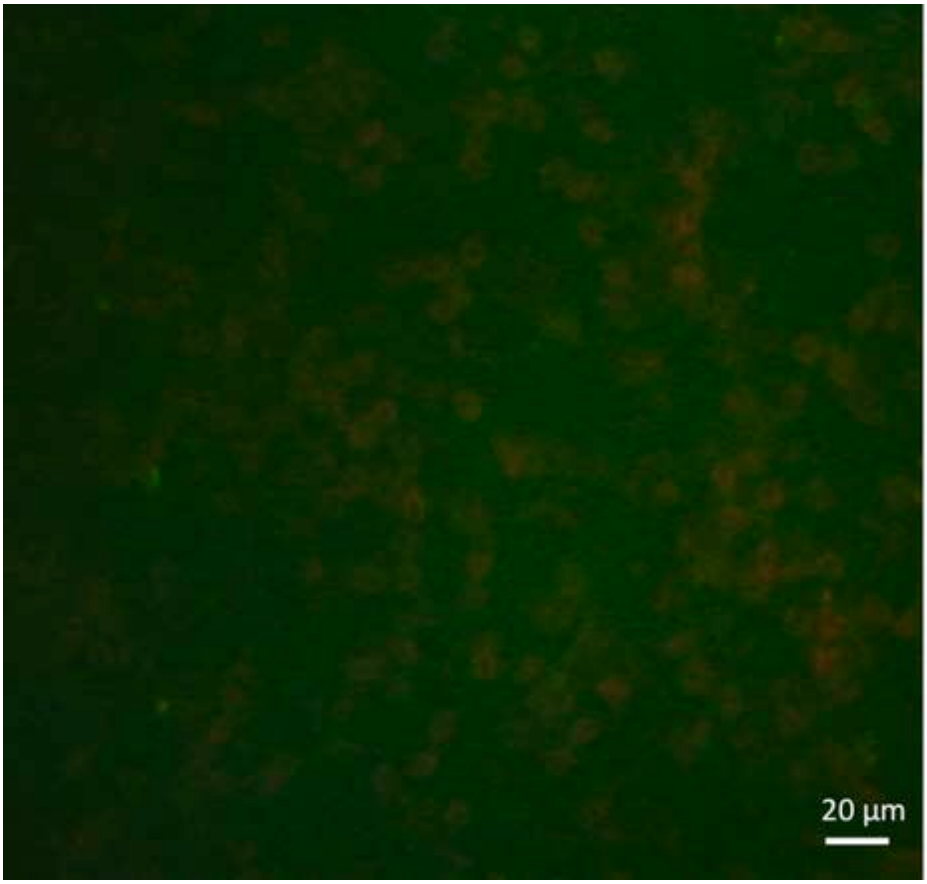

Figure 4.5. Fluorescent micrograph of isolated rainbow trout (*Oncorhynchus mykiss*) leucocytes from control fish. Cells were isolated using a Ficoll gradient and then incubated with a monoclonal antibody (MAb) specific to components of mature spore walls. Cells were then fluorescently labelled with anti-mouse IgG fluorescein isothiocyanate (FITC) and Evan's Blue counterstain. The above image depicts the background autoflourescent appearance of leucocytes from unexposed fish.

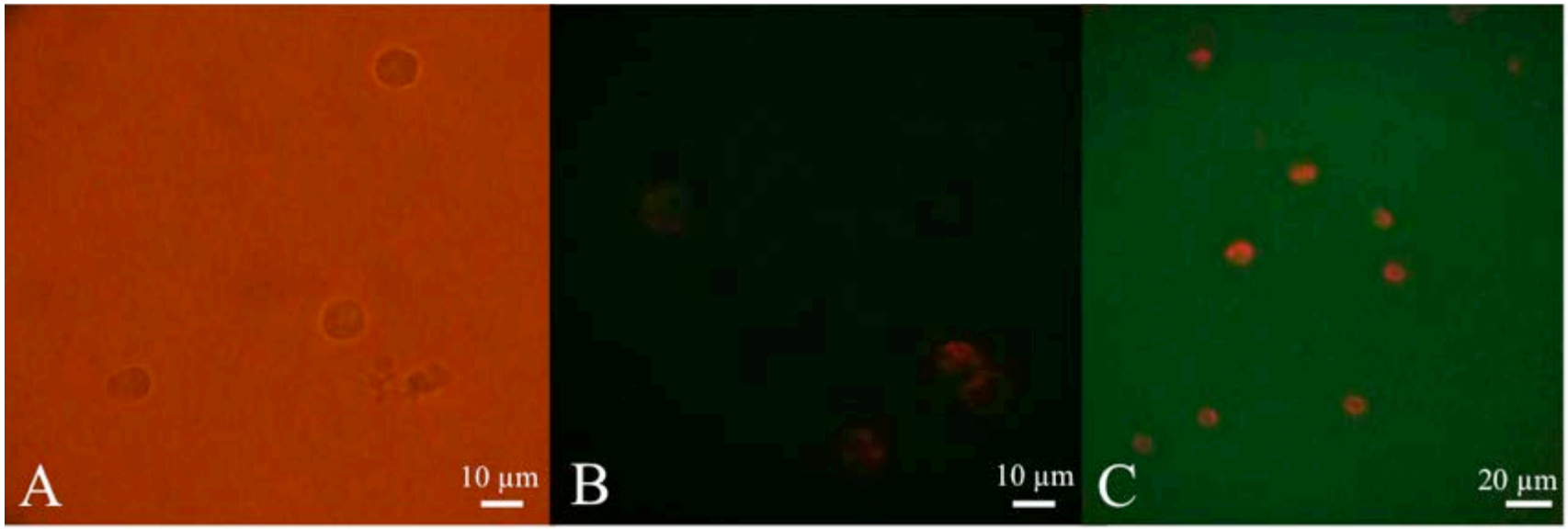

Figure 4.6(A-C). Fluorescent and light micrographs of isolated rainbow trout (*Oncorhynchus mykiss*) leucocytes from fish exposed to *Loma salmonae* infected tissue *per os* for 12 hours. Cells were isolated using a Ficoll gradient and then incubated with a monoclonal antibody (MAb) specific to components of mature spore walls. Cells were then fluorescently labelled with anti-mouse IgG fluorescein isothiocyanate (FITC) and Evan's Blue counterstain. **(A)** Light micrograph depicting the appearance of the isolated leucocytes. **(B)** Fluorescent micrograph depicting the appearance of cells 12 hours post-exposure (PE). **(C)** Fluorescent micrograph depicting leucocytes at a lower magnification, 12 hours PE.

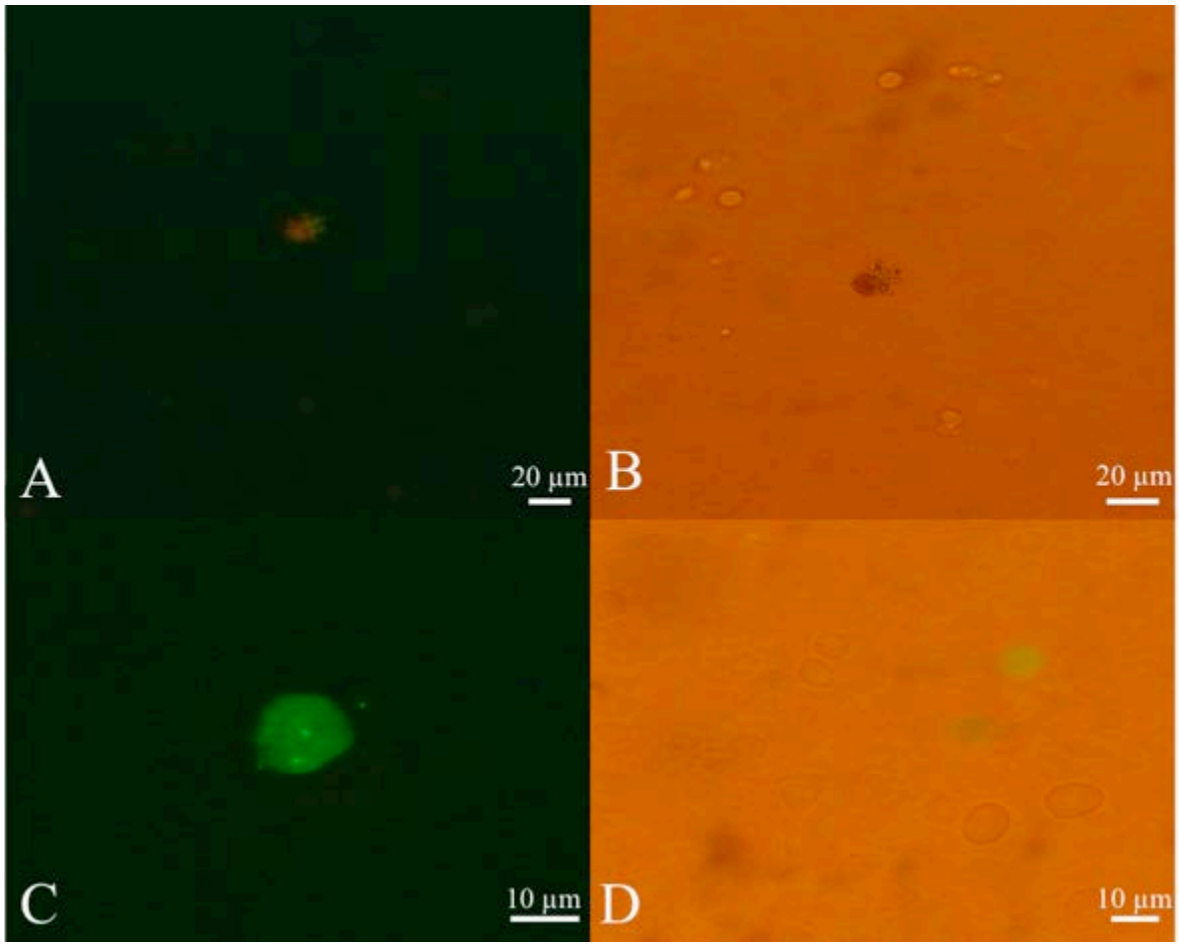

Figure 4.7 (A-D). Fluorescent and light micrographs of isolated rainbow trout leucocytes from fish exposed to *Loma salmonae* infected tissue *per os*, 24 hours post-exposure (PE). **(A)** Granulocytes became more frequent within the isolated cell population 24 hours PE. **(B)** Light micrograph of the same granulocyte seen in image (A). **(C)** Fluorescent micrograph of a diffusely staining FITC-positive cell, containing bright green, granular material. **(D)** Light/fluorescent overlay to depict the strongly fluorescing leucocytes present 24 hours PE.

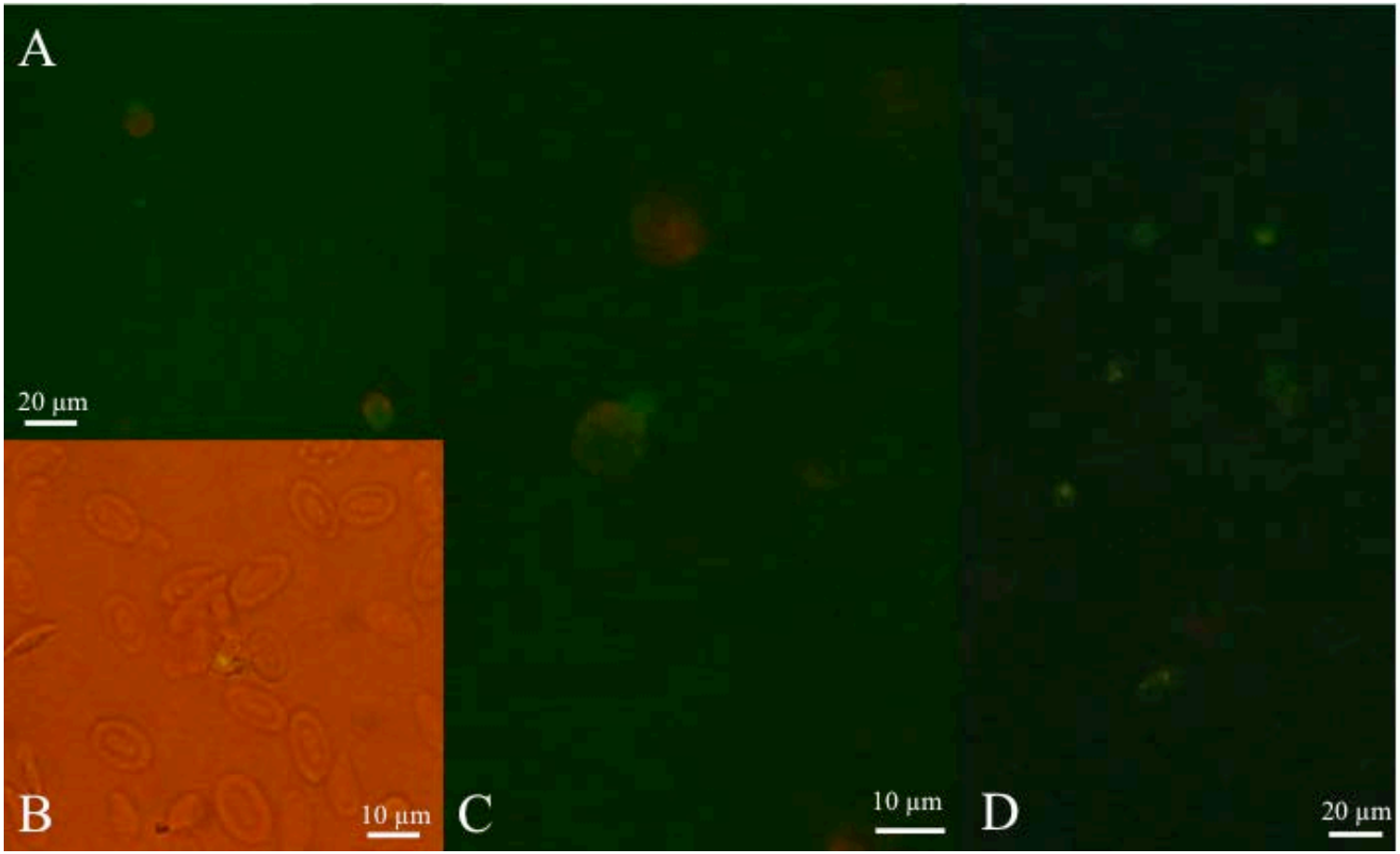

Figure 4.8 (A-D). Fluorescent micrographs of isolated rainbow trout leucocytes from fish exposed to *Loma salmonae* infected tissue *per os*, 1 week post-exposure (PE). **(A)** The majority of leucocytes appear to be fluorescing green (FITC-positive) 1 week, PE. **(B)** Fluorescent/light overlay to depict the appearance of granulocytes within the cell samples. **(C)** High magnification image of brightly fluorescing leucocytes. **(D)** Low magnification fluorescent micrograph of brightly fluorescing cells.

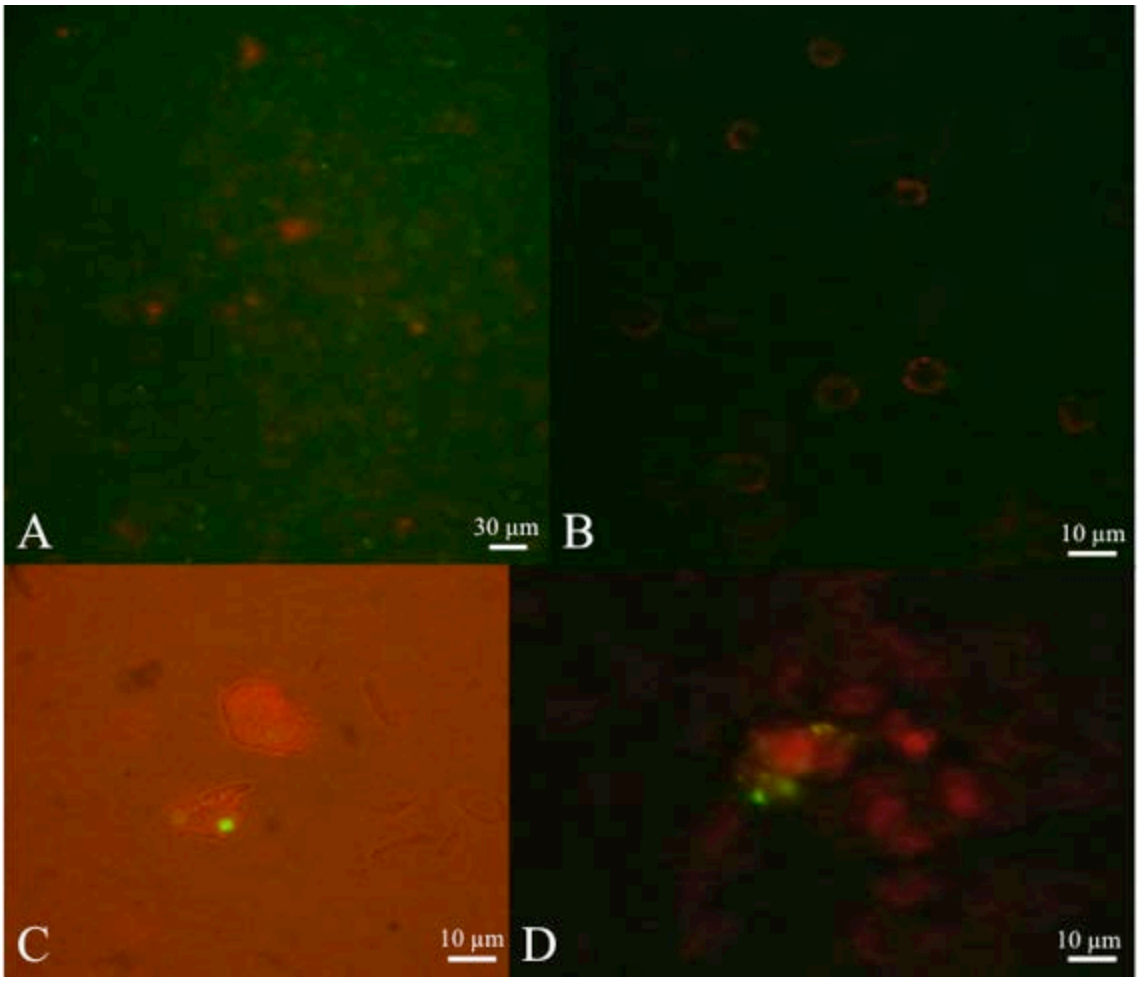

Figure 4.9 (A-D). Fluorescent micrographs of isolated rainbow trout leucocytes from fish exposed to *Loma salmonae* infected tissue *per os*, 2 weeks post-exposure (PE). **(A)** Fluorescent micrograph of the general appearance of the isolated leucocytes 2 weeks PE. **(B)** Higher magnification image of the appearance of isolated leucocytes. The FITC-positive material appears to arrange in clumps attached to, or within cells. **(C)** Fluorescent/light overlay of the appearance of a granulocyte containing bright FITC-positive material, which appear to be within membrane-bound vesicles. **(D)** Fluorescent micrograph of another leucocyte containing granular, FITC-positive material.

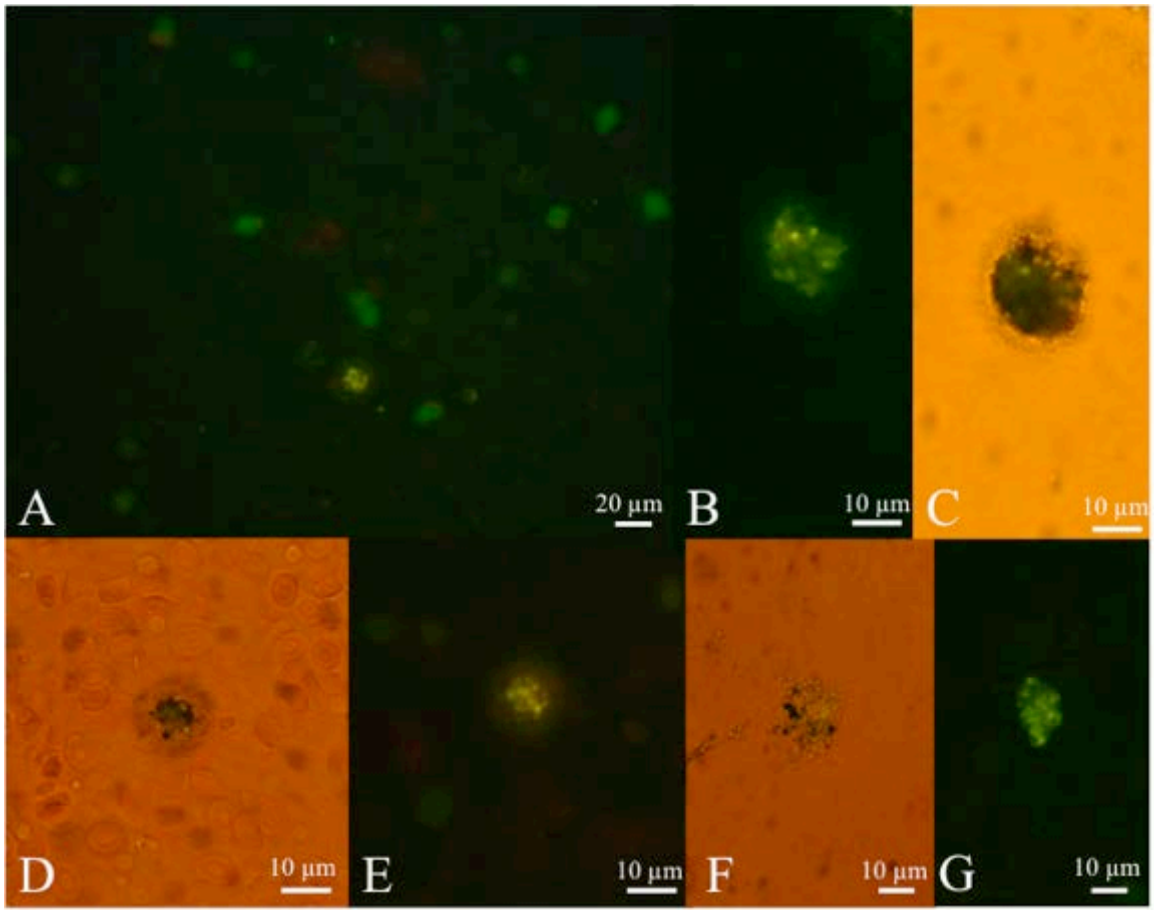

Figure 4.10 (A-G). Fluorescent micrographs of isolated rainbow trout leucocytes from fish exposed to *Loma salmonae* infected tissue *per os*, 3 weeks post-exposure (PE). **(A)** Fluorescent micrograph of the general appearance of the isolated leucocytes. Cells appear to be more brightly fluorescing compared to previous samples. Many granulocytes were detected, containing bright, FITC-positive granules centre of image). **(B)** High magnification fluorescent micrograph of a granulocyte containing bright, FITC-positive granules. **(C)** Light/fluorescent overlay of the granulocyte depicted in (B). **(D)** Light/fluorescent overlay of a granulocyte. **(E)** Fluorescent micrograph of the same granulocyte in (D). **(F)** Light/fluorescent overlay of a granulocyte. **(G)** Fluorescent micrograph of the same granulocyte in (F) highlighting the bright, diffuse nature of the staining 3 weeks PE.

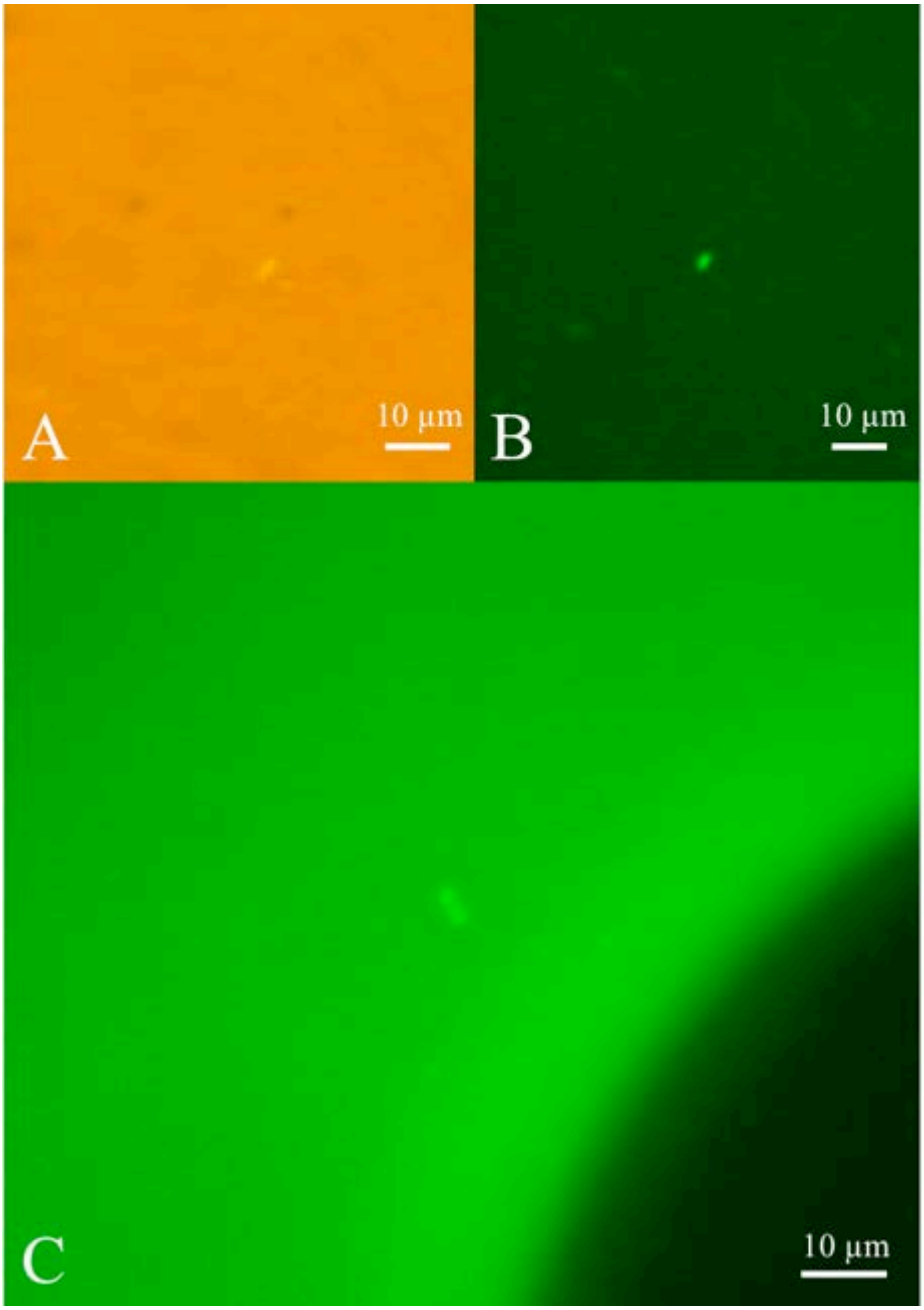

Figure 4.11 (A-C). Fluorescent micrographs of the lysate from isolated rainbow trout leucocytes from fish exposed to *Loma salmonae* infected tissue *per os*, 3 weeks post-exposure (PE). For these images, a subsample of leucocytes were lysed and placed through a Percoll® gradient to remove cellular associations in search for spores and spore-like material. Samples from the lysed cells had significant background fluorescence compared to controls and appeared to contain what looked like empty spores. **(A)** Light/fluorescent over lay of a FITC-positive, spore-shaped particle. **(B)** Fluorescent micrograph of the same particle seen in (A). **(C)** Fluorescent micrograph depicting another spore-like particle with strong background fluorescence.

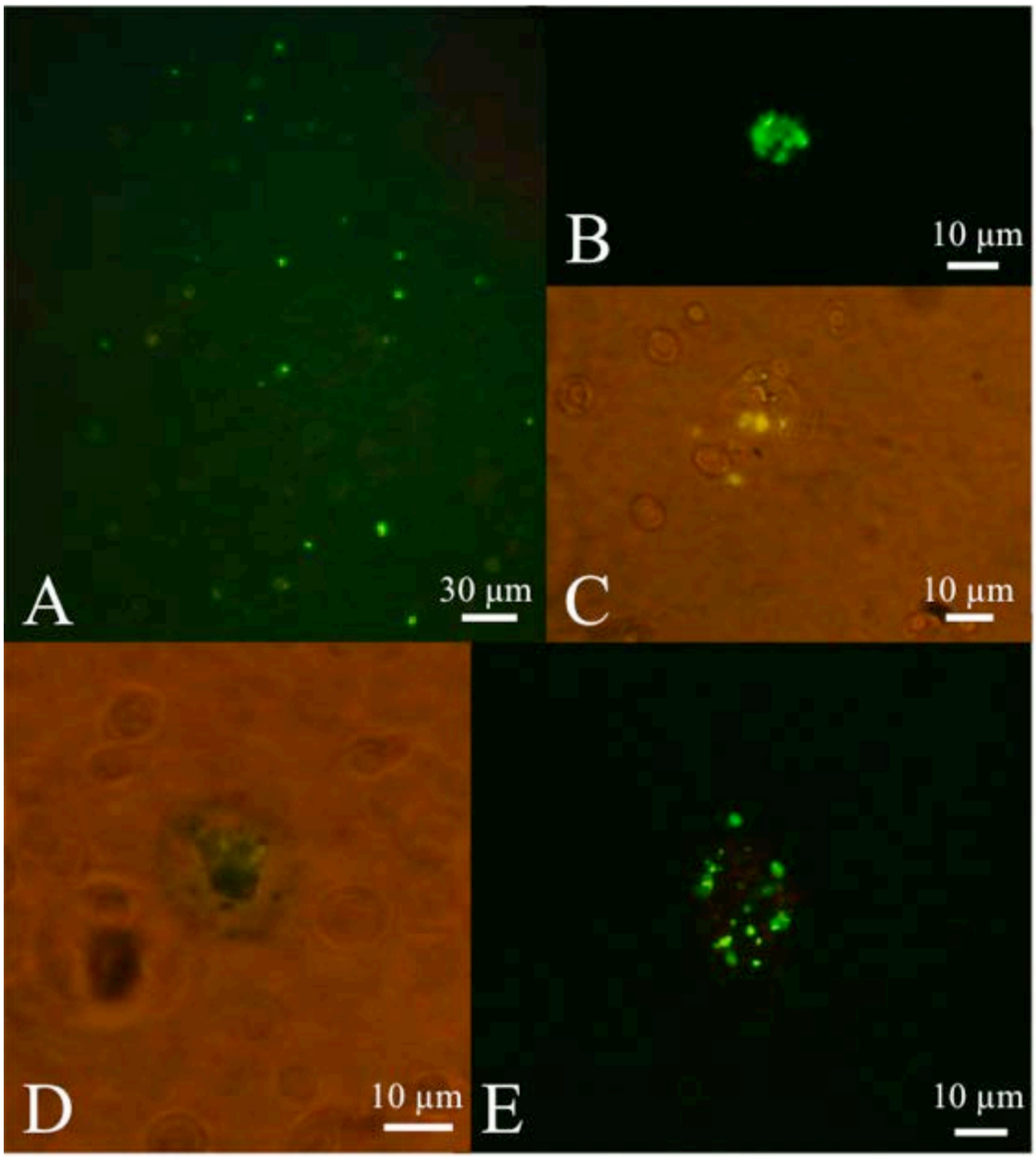

Figure 4.12 (A-E). Fluorescent micrographs of isolated rainbow trout leucocytes from fish exposed to *Loma salmonae* infected tissue *per os*, 4 weeks post-exposure (PE). **(A)** Low magnification fluorescent micrograph depicting the general appearance of the isolated leucocyte 4 weeks PE. **(B)** High magnification fluorescent micrograph of a FITC-positive granulocyte containing brightly staining granules. **(C)** Light/fluorescent overlay image of a similar granulocyte. **(D)** Light/fluorescent overlay image of a similar granulocyte. **(E)** Fluorescent micrograph depicting a FITC-positive granulocyte containing brightly staining granules.

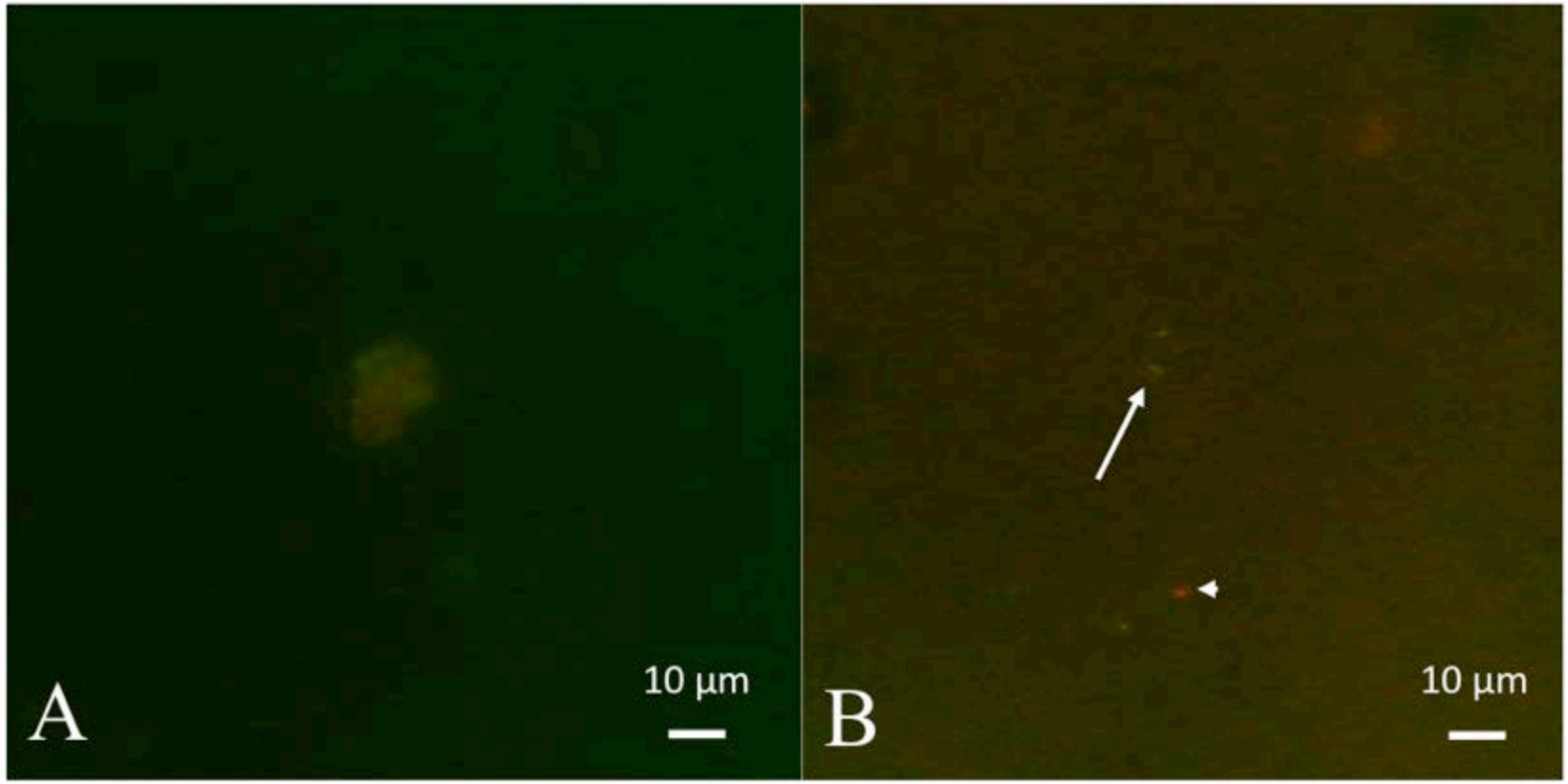

Figure 4.13 (A-B). Fluorescent micrographs of the association of *Loma salmonae* spores with *Aedes albopictus* (AED) cultured cells 10 days post-exposure (PE). Cells were removed from flasks and placed in microcentrifuge tubes. Cells were stained with a monoclonal antibody (MAb), specific to mature *L. salmonae* spores and then fluorescently labelled with anti-mouse IgG fluorescein isothiocyanate (FITC) and propidium iodide (PI). **(A)** Example of the typical appearance of AED cells containing amorphous, FITC-positive material within their cytoplasm. **(B)** Example of the appearance of cells appearing to contain easily distinguishable, FITC-positive spores within cell cytoplasm (arrow). Below the cell is a non-viable spore (arrowhead)

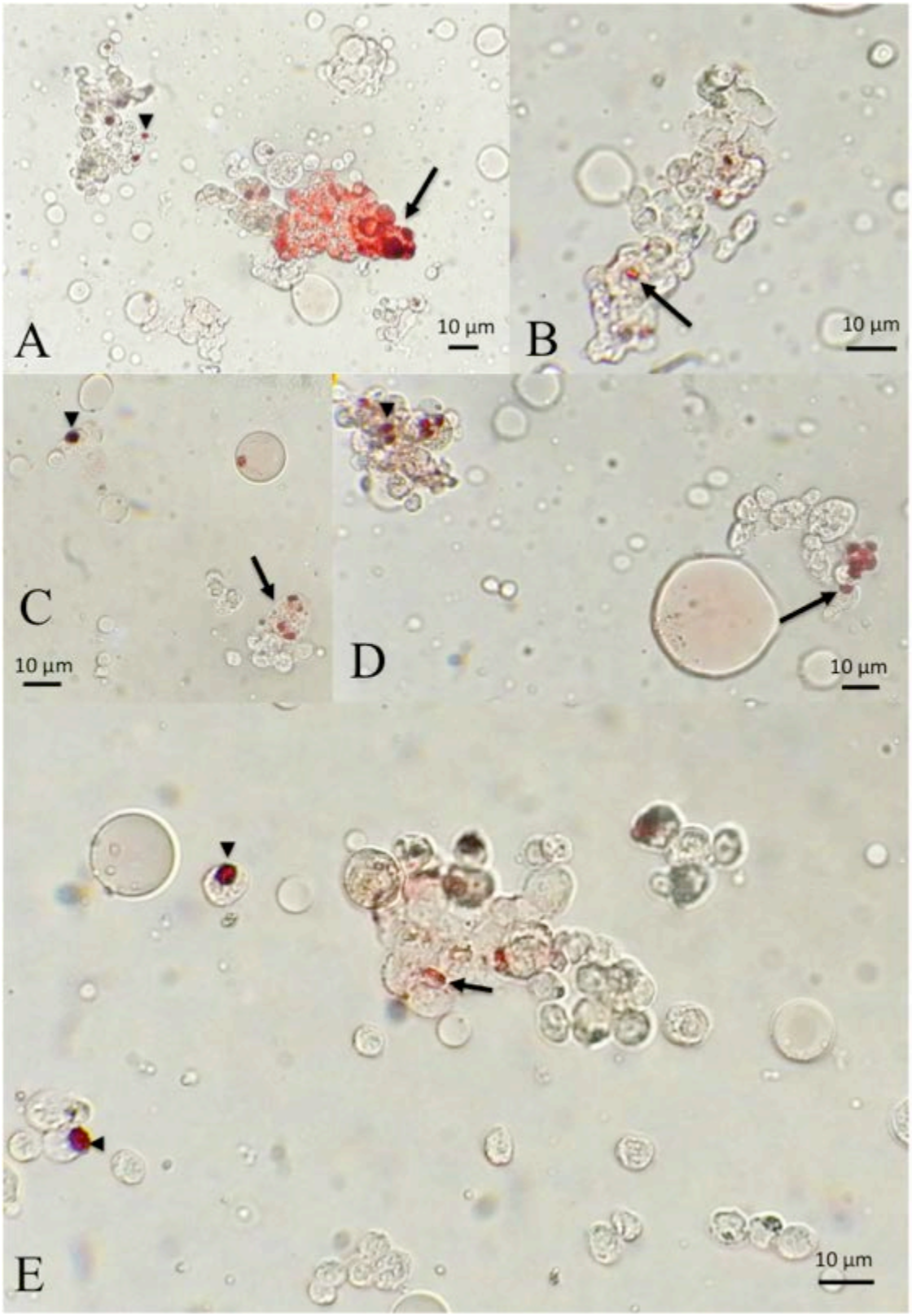
A
10 µm
B
10 µm
C
10 µm
D
10 µm
E
10 µm

Figure 4.14 (A-E): Light micrographs of the association of *Loma salmonae* spores with *Aedes albopictus* (AED) cultured cells 10 days post-exposure (PE). Cells were immunostained with MAb and 3-Amino-9-ethylcarbazole (AEC). **(A)** Often, many AED cells were observed clumped together with large amounts of AEC-positive material dispersed within the clumps. Some spores within the clump appeared to be adhered to outer cell membranes (arrow). Some cells had diffusely pink cytoplasm, while others had single spores within a clear cytoplasm (arrowhead). **(B)** Magnified example of a single spore presumably within the cytoplasm of an AED cell (arrow). **(C)** Further example of easily distinguishable spores apparently within AED cell cytoplasm (arrow). Many cells also appeared to contain very intensely-staining vesicles (arrowhead). Spores within these vesicles were not well defined. **(D)** Further example of spores that appear to be adhered to AED cells (arrow), while others appear to be within cell cytoplasm (arrowhead). **(E)** Higher magnification of an example of well-defined, AEC positive spores within cell cytoplasm, that appears to be weakly AEC-positive (arrow). Also clear in this image is the presence of intensely AEC-positive vesicles within cells containing clear cytoplasm (arrowheads). The AEC-positive material looks as though they may be spores but are quite larger and rounder than typical spores.

4.5 Discussion

Studies in Chapter 3 required developing a method to confidently identify *L. salmonae* spores within mussel excrement and to reliably determine spore viability. The results from Chapter 4 reveal *in vitro* techniques can reliably determine spore presence, quantity, within cells, tissues, and mussel excrement. However, further refinement of the model is needed to differentiate between viable and empty spores. The techniques are also useful in assessing viability of spores following disinfectant techniques.

The MAb-PI *in vitro* assay results agreed with *in vivo* results. There is strong positive correlation between *in vitro* viability and intensity of infection (*i.e.*, xenoma count). Next, several immunocytochemical applications of the MAb protocol were explored. The MAb-PI protocol effectively determines the presence of viable spores within blue mussel feces using fluorescence microscopy. A variation of the MAb protocol involving the immunocytochemical stain AEC effectively visualized spore associations with cultured cells using light microscopy. A MAb-FITC protocol was also developed to observe spore associations with the leucocytes from rainbow trout with an active *L. salmonae* infection. Lastly, the MAb-PI protocol was applied to a suite of chemical disinfectants that have promise in deactivating *L. salmonae* spores, but had never been explored in depth in the context of *in vitro* viability detection. The *in vitro* and *in vivo* studies herein suggest that sodium hypochlorite is the best disinfectant against *L. salmonae* spores.

MAbs are typically produced by clones from a single B cell that recognize a single epitope within an antigen. Consequently, MAbs are useful in identification within mixed and contaminated samples. For example, MAb immunofluoresent antibody tests (IFATs) are routinely used to screen for the presence of human-virulent microsporidia in human

fluids and excrement (Aldras et al., 1994). The Fungi-Fluor® calcofluor white staining method was useful for uniform samples, but was not useful for identifying spores within mussel feces as other fungal spores and bacteria are likely present (*e.g.,* yeast and *Staphyloccocus* spp.). Additionally, the germination protocol using Fungi-Fluor® was not reliable compared to the information provided by using PI. Positive staining with PI is a reliable method that determines membrane integrity. A spore with a compromised membrane is most likely unable to build up the correct osmotic pressure needed for germination, or has already germinated (Frixione et al., 1997; Leiro et al., 1993). However, the PI staining protocol cannot reliably predict whether spores are already empty, or whether intact spores posses the ability to germinate and cause infection. Therefore the viability assay should be used complementary to *in vivo* infectivity. Additional refinement of the *in vitro* viability assay will utilize light microscopy to confirm absence of a posterior vacuole to identify empty, PI-negative spores.

The antigen that the MAb attached to in Chapter 4 has not yet been identified, so this would be a logical next step. Additionally, it would be interesting to perform immunolocalization of the MAb to determine which part of the spore it binds to (*e.g.,* using TEM). It appears that the spore wall is fluorescing during the IFAT procedures, but it would be noteworthy to determine which part of the spore wall is bound. Lastly, formal cross-reactivity studies using the MAb would help determine whether the MAb is specific to *L. salmonae,* or can be used to identify other microsporidian species. Cross-reactivity studies using MAbs obtained from human-infecting microsporidia have had varied results. Some MAbs, such as for *E. cuniculi* have wide reactivity (Niederkorn &

Shadduck, 1980), while others show specificity to only one species (*E. bieneusi*; Accoceberry et al., 1999).

Similarly, the cell isolation procedure appeared successful; however, several modifications could more reliably isolate specific whit blood cell types. In the present study, the protocol isolated monocytes and granulocytes that were present just above the erythrocyte layer in the Ficoll gradient. There are isolation procedures that can further separate leucocyte types and lyse erythrocytes so that they can be observed independently (*e.g.*, Dextran and sodium metrizoate, Oh et al., 2008). Other immunocytochemical techniques, such as flow cytometry could also be useful in cell type separation (Wahl et al., 1984). Infected leucocytes could be processed for TEM as described in Chapter 5. Observing cell spore associations using TEM immunocytochemistry could be useful.

Limited information is available on the effects of disinfectants on microspordian spores, especially for *L. salmonae* (Shaw et al., 2000; John et al., 2005). Therefore, after the *in vitro* assay was verified, several chemical treatments were applied to isolated spores to determine their effectiveness in deactivating spores. Chemical treatments such as sodium hypochlorite, iodine, 70% ethanol and 1% Virkon appeared to be effective disinfectants, whereas formalin and hydrogen peroxide did not appear effective based on the *in vitro* assays. The *in vitro* technique was likely not adequate in determining viability following hydrogen peroxide treatment as it causes germination, which cannot be differentiated from viable spores using the MAb-PI technique. *In vivo* results suggest sodium hypochlorite is the most effective treatment, especially given the high concentration of iodine required to deactivate spores (>300 ppm). This methodology will be useful for the management of *L. salmonae* in a culture scenario. If spores are present

on a farm or in a hatchery, the above methods can help identify the spores and allow managers to quickly verify that their disinfection techniques have been successful. Additionally, viability testing is useful to determine that spores are deactivated prior for use as a whole-spore killed vaccine. A vaccine is not yet in use, but treatments for *L. salmonae* and MGDS are generally not effective (Becker et al., 2002). A whole-spore killed vaccine has been shown to be effective and has potential utility in fish health management (Harkness et al., 2013). If it is developed, the *in vitro* viability assay would be useful.

4.6 References

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Chapter 5.0 EXPANDING THE *LOMA SALMONAE* DISEASE MODEL: INTERACTIONS WITH CULTURED CELLS[1]

5.1 Abstract

Growth and propagation of fish-infecting microsporidians within cell culture has been more difficult to achieve than for insect- and human-infecting microsporidians. Fish microsporidia tend to elicit xenoma development rather than diffuse growth *in vivo*, and this process likely increases host specificity. The growth of the fish microsporidian, *Loma salmonae*, was attempted with three cell lines: rainbow trout gill epithelial (RTgill-W1), Chinook salmon embryo (CHSE-214), and mosquito *Aedes albopictus* (AED) cells. It was determined that *L. salmonae* has the capacity to develop xenomas within the RTgill-W1 cell line. Spore numbers increased over a 4 week period within cell culture flasks. Xenoma-like structures were observed using phase contrast microscopy, and then confirmed using transmission electron microscopy. No evidence of parasite development was observed in CHSE or AED cells. However, AED cells rapidly phagocytize spores while retaining viability and infectivity in rainbow trout (*Oncorhynchus mykiss*). AED cells were utilized as a method to cryopreserve spores for storage; preliminary studies revealed spores retained *in vivo* infectivity. Optimization of the *L. salmonae*-RTgill-W1 cell model has important implications in elucidating the process of xenoma development induced by microsporidian parasites. Similarly, the use of AED cells as a cryoprotectant will help maintain and diversify the *L. salmonae* disease model.

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[1]McConnachie, S. H., Sheppard, J., Wright, G. M., & Speare, D. J. (2015). Development of the microsporidian parasite, *Loma salmonae*, in a rainbow trout gill epithelial cell line (RTG-1): evidence of xenoma development *in vitro*. *Parasitology*, *142*(02), 326-331.

5.2 Introduction

Microsporidian parasites are obligate intracellular pathogens, currently classified as Fungi (James et al., 2013), that can parasitize most invertebrates and vertebrates (Canning & Vávra, 2000), often causing severe disease and population collapse (*e.g.*, European honeybees; Williams et al., 2008). The culture of parasitic microsporidians has ongoing success for many insect- and human-infecting microsporidians (Monaghan et al., 2009). *In vitro*, some microsporidians complete their life cycle and produce infective spores at production levels for identification, experimentation, preliminary drug screening, and biocontrol (*e.g.*, *Paranosema locustae*; Sokolova et al., 2003) (Monaghan et al., 2009).

Culturing fish-infecting microsporidians has had some success, although not at the level achieved with human- and insect-infecting microsporidians (Monaghan et al., 2009). Successful development has mostly occurred within primary cultures. For example, the salmonid parasite, *Nucleospora salmonis*, has been successfully cultured and propagated within the nuclei of leucocytes harvested from Chinook salmon (*Oncorhynchus tshawytscha*) blood (Wongtavatchai et al., 1995) and kidney from Chinook salmon and rainbow trout (*O. mykiss*; Desportes-Livage et al., 1996). *Heterosporis anguillarum* meronts develop within the Japanese eel (EP-1) cell line; cells are infective to eels, although mature spores have not been detected (Kou et al., 1995). Similarly, Lores and colleagues (2003) saw low levels of development of *Glugea* sp. in the *Aedes albopictus* (AED) mosquito cell line, and development within Chinook salmon embryo (CHSE-214) cells that stopped after 48 hours. The growth of *Pseudoloma neurophilia* has been attempted in several cell lines, but few spores have been detected

within cells (Watral et al., 2006). Preliminary studies with a cod larvae-derived cell line (GML-5) indicate that cells support the growth of *Loma morhua* (McLeod, 2012). The only successful immortal cell culture model that supports the development and propagation of fish-infecting microsporidians has been the brushtooth lizardfish (*Saurida undosquamis*) infecting species, *Heterosporis saurida* in eel kidney cells (EK-1) (Saleh et al., 2014).

The challenge may be that fish microsporidians have higher host specificity in many of the broadly-infecting insect microsporidia. One factor for increased host-specificity in fish could be that most microsporidians develop diffusely within the host body cells, but several species infect cells and transform the host cells into hypertrophic cell-parasite complexes termed xenomas (Lom & Dyková, 2005). Only a few microsporidian species cause xenoma development - the majority being species that infect fish (Lom & Dyková, 2005). Xenomas are always single host cells that are hypertrophic compared to surrounding cells, and contain all life stages of the parasite. They provide optimal growth conditions for developing microsporidians; their structure varies based on the species and its stage of development (Lom & Dyková, 2005).

A xenoma-forming fish microsporidian that is of particular interest is *L. salmonae*, the causative agent of Microsporidial Gill Disease of Salmon (MGDS). *L. salmonae* xenomas develop within gill tissue and elicit chronic inflammatory branchitis and subsequent respiratory distress (Speare & Lovy, 2012). A rainbow trout-*L. salmonae* disease transmission model successfully propagates spores for use in experiments studying the pathogenicity, immunology, and life cycle characteristics of this pathogen (Speare & Lovy, 2012). Several limitations of the *in vivo* model include a 6-week waiting

period for the infection to mature to propagate spores and as such, reliable drug screening cannot be effectively completed, and understanding the early developmental biology of xenomas cannot be easily done.

Of great interest is the development of fish-infecting microsporidians in cell culture, as they often cause high mortality rates in economically important species (Shaw et al., 1999; Becker & Speare, 2007). Currently, no *in vitro* model supporting xenoma-forming microsporidia is available to show how xenomas occur and whether microsporidians can develop into a xenoma when the cell is not influenced bythe host organism (Lom & Dyková, 2005). It would be beneficial to develop *L. salmonae* at production levels *in vitro* to reduce fish numbers used in the disease model, and to propagate spores for vaccine use. The purpose of the present study was to determine if *L. salmonae* could be grown *in vitro*. Three different cell lines were compared, each chosen for their potential from previous studies (AED and CHSE-214 cells; Lores et al., 2003), or for their similarity to *L. salmonae* host tissue (rainbow trout gill epithelial cells, RTgill-W1). During experimentation it was noted that AED cells rapidly phagocytize spores. The use of AED cells as a cryoprotectant to help store the *L. salmonae* disease model was also investigated as spores do not appear to maintain viability after freezing (Shaw et al., 2000; Table 5.1).

5.3 Materials and Methods

5.3.1 Source of spores

Spores were processed using the methods described by McConnachie et al. (2013). Briefly, rainbow trout heavily infected with *L. salmonae* xenomas were euthanized by an overdose of benzocaine (120 mg L^{-1}; Sigma-Aldrich). The gills were immediately dissected from the fish and placed in sterile saline containing 2 mg of penicillin/streptomycin and 100 µg gentamicin mL^{-1}. Gills were homogenized using a tissue grinder, pushed through a cell dissociation sieve, 20 µm Nytex® mesh and then rinsed with saline. The material was centrifuged (350 xg) and the pellet was re-suspended in 20 mL sterile distilled water and vortexed with an equal volume of Percoll®. The mixture was centrifuged (~1000 xg) and the spores pelleted at the bottom of the tube while the remaining cellular debris was removed in the Percoll®-water layers. The cells were washed and re-suspended in sterile saline containing 0.1 mg units of penicillin/streptomycin and 50 µg gentamicin mL^{-1} until further use. Spores were used within 14 days after being purified.

5.3.2 Maintenance of cell lines

RTgill-W1, CHSE-214 and AED cells were originally obtained from the American Type Culture Collection (ATCC) and grown at 20°C. Cells were maintained in L-15 (Leibovitz) medium with 2 mM L-glutamine, 10% fetal bovine serum (FBS) and 100 IU mL^{-1} penicillin/streptomycin (henceforth known as complete medium). Cells were kept in 75 cm^2 flasks at ~75% confluence until experimentation. Cells were split and placed into 6-well plates prior to experimentation. Cells were allowed to reach ~80% confluence (~1.0 x 10^6 cells) before spore inoculation. Both CHSE-214 and RTgill-W1

cells are adherent cells, while AED cells are semi-adherent, with many cells developing free within the media.

5.3.3 Production of spores within cell lines

Spores were centrifuged and re-suspended in complete medium. Spore concentrations were then estimated using a hemocytometer, and spore viability was verified using an in-house developed (J. Sheppard; Speare et al., 1998) monoclonal antibody/propidium iodine (MAb-PI) dye exclusion test (see Chapter 4). Spore concentrations were adjusted to ensure that a ratio of 10:1 spores to cells was plated onto the cells (Monaghan, 2011). Approximately 1.0×10^7 spores were placed into each well. Control wells were maintained. Spores were left on the plates for 3 days and then removed; the monolayer was rinsed with complete media and then re-fed with complete medium. Otherwise, media was replaced weekly. For the semi-adherent AED cells, the media was gently pipetted from the well and then replaced with fresh media.

5.3.4 Detection of spore development

To determine whether spores were developing in the cell lines, a separate experiment was designed. Cells of each type were plated in 12.5 cm^2 (Falcon) and 75 cm^2 flasks and allowed to reach ~80% confluence. Spores were plated on the 12.5 cm^2 flasks at a 10:1 spore to cell ratio. Spores were left on the cell monolayer for 3 days, removed within the supernatant, centrifuged (350 xg) and re-suspended in complete medium. The re-suspended spores were counted using a hemocytometer in order to determine an estimation of spore concentration, and placed back into the 12.5 cm^2 flasks on the monolayer. After 7 days, the spores were removed, centrifuged (350 xg), re-suspended in

complete medium, counted and placed onto a 75 cm^2 flask with the same cell type. Twice a week, spores were removed from the 12.5 cm^2 flasks, centrifuged (350 xg), re-suspended, counted and placed back on to the corresponding 75 cm^2 flask for up to 4 weeks. Spores in the corresponding 12.5 cm^2 and 75 cm^2 flasks were counted once a week and re-plated. Control flasks were maintained and 2 replicates were completed. AED cells appeared to rapidly phagocytize spores from the media and thus could not be counted each week. Mean spore numbers produced each week ± SE were determined using JMP10 software (SAS Institute, Cary, NC).

5.3.5 Observation of spore-cell associations using light and transmission electron microscopy

Cells on the 6-well plates were observed using bright field and phase contrast light microscopy on an inverted microscope. Previous pilot studies suggested that spore production became noticeable around 10 days post-exposure (PE), and thus samples were removed and processed for transmission electron microscopy (TEM) at 10 days and then weekly for up to 4 weeks. Samples from RTgill-W1 cells were also taken 5 days PE. Control cells were sampled at each time point. Cells were removed from the flask by pipetting the media gently and were placed into 2% glutaraldehyde (EM-grade, Canemco & Merivac) in 0.1M phosphate buffer (pH 7.2). Cells that did not lift by agitation were rinsed with versene (Gibco®), phosphate buffered saline and then exposed to TrypLE (Gibco®) for ~ 1 minute. The TrypLE was deactivated with complete media and the mixture was added to the glutaraldehyde solution. Cells were fixed at room temperature (~25° C), for 1 hour, centrifuged and re-suspended in fresh 2% glutaraldehyde in 0.1M phosphate buffer and left for ~12 hours. Cells were spun down (~4000 xg) and pellets

were washed using phosphate buffer, post-fixed in phosphate buffered 1% osmium tetroxide (Canemco & Merivac) for 1 hour and then placed in agar. After several dehydration steps using ethanol and propylene oxide, cell pellets were embedded in Spurr's resin. After resin polymerization, semi-thin (0.5 µm thick) sections were cut and stained with 1% toluidine blue in 1% sodium tetraborate (Sigma-Aldrich) to observe the cells using light microscopy. When sections containing spores or signs of cellular hypertrophy were observed, thin sections (70-90 nm thick) were cut and stained with uranyl acetate (depleted uranium; Canemco & Merivac), and lead citrate (Canemco & Merivac). Stained thin sections were examined using a Hitachi H7500 TEM (BioTEM, Nissei-Sangyo) operated at 80 kV. Images were captured using the AMTV600 digital image capture engine and an AMT XR 40 digital camera (Danvers, MA, USA).

5.3.6 Utilization of AED cells as a cryoprotectant for L. salmonae spores

After it was determined that AED cells rapidly phagocytize *L. salmonae* spores within 3 days, an experiment was designed to determine whether cells could be used as a cryoprotectant for the disease model. A previous pilot study revealed that storing spores in 10% FBS and 90% DMSO (freezing media) did not preserve *in vivo* viability (Table 5.1). 10 million spores were purified and aliquoted into 2, 50 mL tubes, centrifuged (~350 xg) to remove the sterile saline and re-suspended in 50 million AED cells per tube in complete media at a ratio of 10 cells to 1 spore. Spores and cells were transferred to 12.5 cm^2 flasks and left at room temperature (~25° C), for 48 hours, until very few spores remained free in the media. AED cells were agitated and removed from the flasks, centrifuged, and placed in 3 mL cryotubes with 1.5 mL of freezing media. Subsamples of spores for viability analysis were taken and compared with spores at inoculation using the

MAb-PI test. Spores were then placed in a -80°C freezer for 24 hours for gradual freezing and were then stored in liquid nitrogen.

Six months later, the samples were thawed, washed and re-suspended in complete media. A subsample of each tube was taken to asses the viability of the AED cells using 50% Trypan blue, and the spores using the MAb-PI test. The tubes containing spores within cells were processed into gelatin (Chapter 2) and left to solidify for 1 hour. The samples were minced and fed to naïve rainbow trout at a dose of ~500,000 spores per fish in 2 separate tanks. In another tank, naïve fish were fed a sample of purified spores processed into gelatin feed as a positive control. In yet another tank, fish were anesthetized, fin-clipped and IP injected with saline (negative control) and 500,000 purified spores per fish (positive control). After 24 hours, the fish fed gelatin were anesthetized, fin-clipped and placed in the tank with the controls.

Fish were maintained for 6 weeks to allow infections with *L. salmonae* to mature (Chapter 2). Fish were then euthanized and the first left gill arch of each fish was removed and observed under a microscope. Infection status and the number of spore-filled xenomas were tabulated and compared among treatment groups (% infected and mean xenoma count).

Lastly, 2, 12.5 cm^2 flasks of AED cells were inoculated with purified spores at a ratio of 1 cell to 10 spores. Spores and cells were randomly removed from the flasks and processed for TEM to determine the state of spores within AED cells shortly following phagocytosis. Samples were taken at 6 hours, 1-5 days, 10 days, 2 and 3 weeks PE.

5.4 Results

5.4.1 Appearance of control cells

Control (uninfected) RTgill-W1 cells had typical, discoid, epithelial morphology and formed adherent monolayers (Figure 5.1A). Cells often had attenuated cytoplasmic processes and typical, spherical nuclei (Figures 5.1A-B). Secondary lysosomes containing material phagocytized from the media (*e.g.*, dead cells and membranous components) were commonly observed within control and spore-exposed cells (Figure 5.1C).

Control CHSE-214 cells also appeared as discoid, epithelial-like cells. However, cells were typically larger, more rounded, and contained phase-bright granules within their cytoplasm (Figure 5.2A). Cells adhered to the culture flasks quite firmly compared to RTgill-W1 cells. Ultrastructurally there were lobulated nuclei, frequent secondary lysosomes, and large vesicles containing electron-dense material (Figures 5.2B-C).

AED cells did not adhere well to the culture flasks and multiplied very rapidly compared to the other cell types. Cells were small and round, and often contained large, phase-bright vacuole-like structures (Figures 5.3A-B). Cells rapidly phagocytized any debris within the media, including dead AED cells (Figure 5.3C). AED cells contained prominent rough endoplasmic reticulum (RER), many mitochondria, and membrane-bound vesicles containing various densities and sizes of membranous and granular material (Figures 5.3D-F).

5.4.2 Production of spores within RTgill-W1 and CHSE-214 Cells

In RTgill-W1 cells, spores were being produced at 1week PE, and a steady increase in spore numbers was observed up to 4 weeks (Figure 5.4). By 3 days PE, several cells appeared hypertrophic and darker than other cells. Using phase-contrast microscopy, spores were evident within the hypertrophic cells by 5 days PE (Figure 5.5A). The hypertrophic cells had spores associated with their surface and many phase-dark spores packed within their cytoplasm surrounding the nucleus (Figure 5.5A). Approximately 10 days PE, after spores had been washed from the monolayer, many spores were still observed on the monolayer. The monolayer became ragged and easily detachable by 1 week, but appeared to recover (become adherent) by 4 weeks. After 4 weeks the number of spores decreased in the flasks.

One week PE, spore numbers within CHSE-214 decreased, with spore numbers reduced by more than 200,000 spores (Figure 5.4). In weeks 2-4, spore numbers increased, but never exceeded the inoculation dose. Spores appeared to disrupt CHSE-214 cell monolayers, with most cells becoming detached by 1 week. Monolayers eventually recovered by 3-4 weeks. Often, using phase-contrast microscopy, spores appeared to be present within CHSE-214 cells, but not in the arrangements and density commonly seen in RTgill-W1 cells (*i.e.,* Figure 5.5A).

5.4.3 Observation of spore- RTgill-W1 cell associations using TEM

Serial, ultrastructural examination of a hypertrophic, spore filled cell, first observed in a toluidine stained semi-thin section (10 days PE; Figure 5.6) revealed the structure was a xenoma containing mostly intact mature spores that demonstrated the characteristic 15-17 polar tube coils (Figures 5.5B-C). The xenoma host cell was much

larger than the surrounding, non-infected cells. Many mature spores, empty spores and various, degraded parasite developmental stages were present within large irregularly-shaped parasitic vacuoles throughout the cytoplasm of the xenoma (Figures 5.5C-D; Figures 5.7A-D). The membranes of the degraded developmental stages were dissimilar to mature spores, electron-dense, and the cytoplasm contained disorganized, membranous and amorphous material (Figures 5.7A-C). The cytoplasm of the xenoma appeared to be less electron dense compared to surrounding cells, and portions of the peripheral cytoplasm and surrounding plasma membrane of the xenoma exhibited blebbing (Figure 5.5D). Tubular elements were evident within parasitic vacuoles containing mature and empty spores (Figure 5.5D: Figure 5.7A).

Several cells contained membrane-bound structures that were considered to be *L. salmonae* meronts (Figure 5.8). The structures within the membranes were more electron-lucent than the surrounding cytoplasm and were surrounded by a membrane that often appeared double-layered (Figure 5.8C). The structures were often closely associated with host-cell mitochondria (Figure 5.8B).

Overall, intact spores were found surrounding the cells with no cellular associations. Several cells did contain single, intact spores within their cytoplasm that were often associated with secondary lysosomes and some of these spores appeared degraded with loss in electron density (Figures 5.9A-C).

5.4.4 Observation of spore-CHSE-214 cell associations using TEM

In CHSE-214 cells, intact spores were commonly found surrounding cells, without any cellular associations. Many cells did contain one to many spores within their cytoplasm (5.10A), and were generally associated with secondary lysosomes (Figure

5.10B). Spores within secondary lysosomes commonly had less electron density and often appeared degraded (Figure 5.10B).

5.4.5 Observation of spore-AED cell associations using phase-contrast microscopy and TEM

Spores disappeared from the media in exposed AED cell suspensions within 3 days. Within 1 day spores appeared to make contact with AED cells (Figure 5.11A). By day 2, very few cells were free within the media. Most spores appeared to make contact with cell membranes, or were within the cells. By 3 days, many cells became enlarged and filled with spores. Very few spores remained free within the media by day 3.

TEM micrographs taken 10 days and 2 weeks PE demonstrated intact spores within cells, or associated with cell membranes (Figure 5.12). Intact spores often were in contact with projecting pseudopodia from AED cells (Figures 5.12A-B). Several spores were also seen within incomplete phagosomes (Figure 5.12B). Intact spores within cells were usually associated with large, membrane-bound vesicles containing sparse granular material (Figures 5.12D-F). The granular material appeared to adhere to the surface of spores (Figure 5.12F). Spores were also associated with membrane-bound vesicles containing densely packed, electron-dense granular material, which appeared to be secondary lysosomes (Figures 5.12G-I). Spores within these vesicles often appeared degraded (Figure 5.12H) and empty (Figure 5.12I).

5.4.6 Utilization of AED cells as a cryoprotectant for L. salmonae spores

Prior to phagocytosis by AED cells, analysis by MAb-PI yielded a spore viability of 85%. Spores became associated with the outer surface of AED cells by at least 1 hour after exposure (Figure 5.13A). By 3 hours, most cells were associated with large

aggregates of phase-bright spores, often in contact with cell surfaces (Figure 5.13B). After 24 hours, many cells appeared enlarged and distended with phase-dark spores (Figure 5.13C). After exposure to AED cells for 48 hours, and prior to freezing, spore viability decreased to 76%. TEM micrographs taken at regular intervals from 6 hours PE to 3 weeks PE revealed that the majority of spores remained intact for up to 3 weeks PE. Spores were seen within the cytoplasm of AED cells 6 hours PE, although many cells were free of cellular associations (Figure 5.14A). One day PE onward, most spores were found within cells, but mostly remained intact (Figures 5.12B-I). Spores were commonly associated with membrane-bound vesicles filled with varying densities of granular material (Figures 5.14A-E,G-I). Spores within incomplete phagosomes (*i.e.*, actively being phagocytized) were seen at all sample points (Figure 5.14F). A higher proportion of degraded and empty spores were seen from 10 days PE onwards (Figure 5.15). Degraded spores appeared to be within secondary lysosomes and granular material was often associated with components of degrading spores (Figures 5.15A-D).

Six months later, spores that were thawed and processed in gelatin feed were 27.5% viable. Approximately 64% of cells maintained viability after freezing. Six-weeks after exposure to gelatin-processed spores and AED cells, 43% of exposed fish were infected, with an average of 6 (±2) xenomas per left gill arch. 90% IP-injected, positive control fish, and 70% of gelatin-positive control fish had xenomas (average per first left gill arch, 11 (±3) and 5 (±2), respectively). No negative control fish were infected at 6 weeks. (Table 5.2)

Table 5.1. Summary of results for the *in vivo* infectivity of cryopreserved *Loma salmonae* spores stored in Leibovitz-15 media and 10% DMSO (freezing media) for 2 weeks, 3, and 12 months. After thawing, spores were washed and re-suspended in sterile saline and injected into naïve rainbow trout (*Oncorhynchus mykiss*) intraperitoneally (IP). Fish were euthanized 6 weeks post-exposure and the proportion of fish infected and the number of xenomas present (mean ± standard error, SE) on the first left gill arch were tabulated.

	Proportion with Xenomas on Gills	Mean Xenoma Count on 1st Left Gill Arch in Infected Fish (±SE)
2 Weeks		
Negative Control	0/20	0
Positive Control	8/8	34±8
IP Injected Fish	2/45	4±2
3 Months		
Negative Control	0/5	0
Positive Control	4/5	6±2
IP Injected Fish	5/14	8±4
12 Months		
Negative Control	0/5	0
Positive Control	4/4	33±13
IP Injected Fish	2/18	1±0.1

Table 5.2. Summary of results for the *in vivo* trials involving *L. salmonae* spores that had been cryopreserved in freezing media after being phagocytized by *Aedes albopictus* (AED) cells. Samples were thawed after 6 months and processed into a gelatin medium that was then fed to naïve rainbow trout. Fish were euthanized 6 weeks post-exposure and the proportion of fish infected and the number of xenomas present (mean ± standard error, SE) on the first left gill arch were tabulated.

	Proportion with Xenomas on Gills	Mean Xenoma Count on 1st Left Gill Arch in Infected Fish (±SE)
Spores and Cells in Gelatin		
Negative Control	0/5	0
IP Injected Positive Control	9/10	11±3
Gelatin and Control Spores	7/10	5±2
Gelatin and Frozen Spore-Fed Fish	9/21	6±2

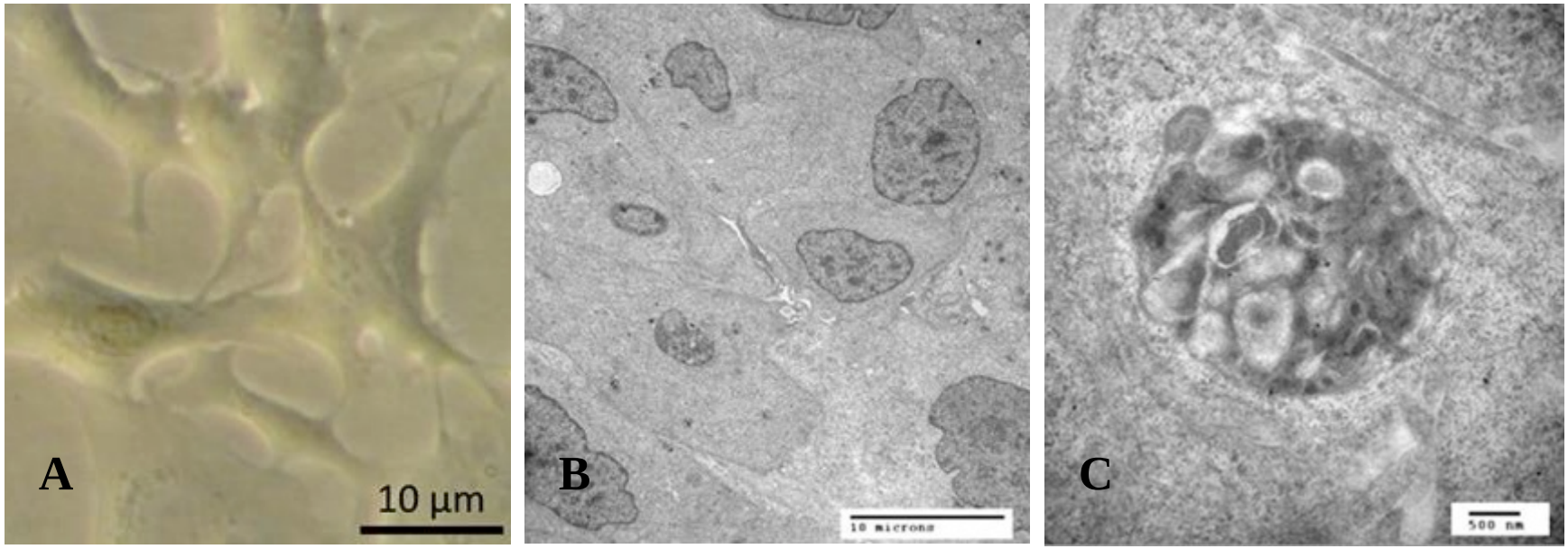

Figure 5.1 (A-C): Control rainbow trout gill epithelial cells (RTgill-W1), 2 weeks post-plating. **(A)** Phase-contrast light microscopy image of epithelial-like cells forming a monolayer in $L15^{+}$ media. The attenuated cytoplasmic processes are especially pronounced. **(B)** TEM micrograph of an adhered monolayer of cells. Cells contain typical spherical nuclei and are closely apposed, often forming tight junctions and desmosomes. **(C)** High magnification TEM image of a secondary lysosome within the cytoplasm, depicting their typical appearance.

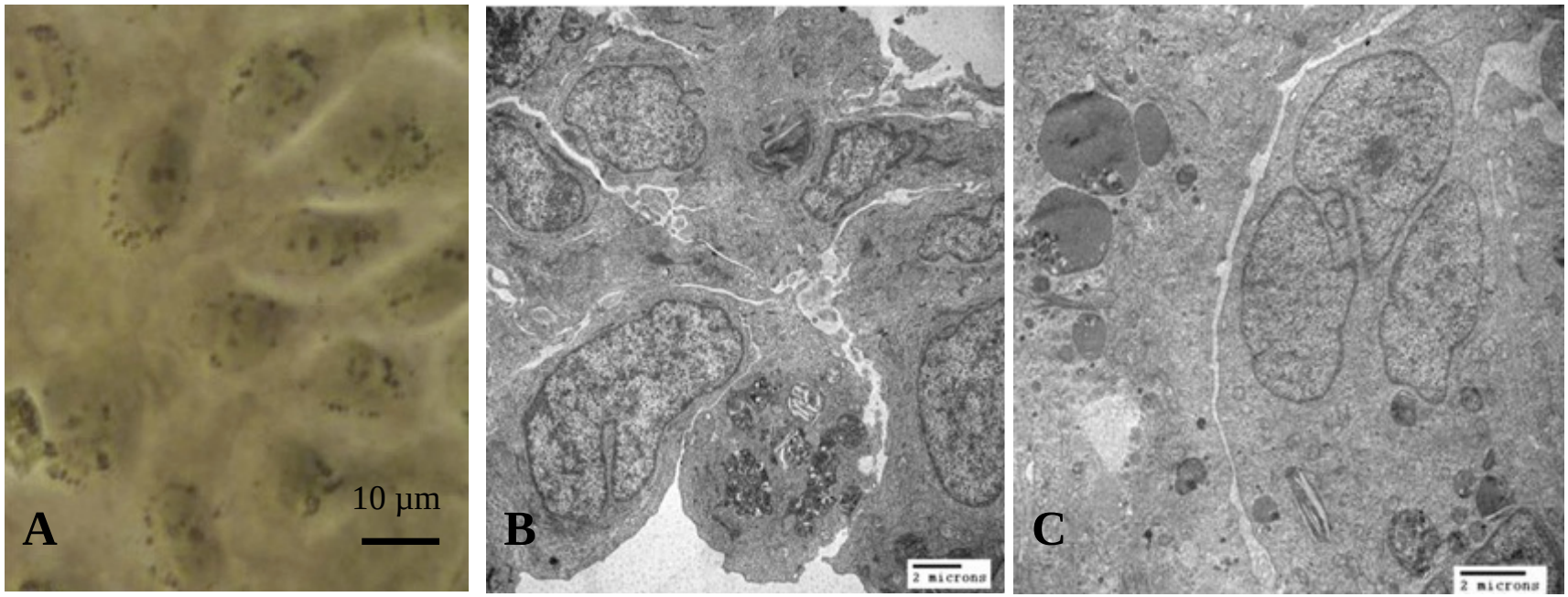

Figure 5.2 (A-C): Control Chinook salmon embryo (CHSE-214) cells 2 weeks post-plating. **(A)** Phase-contrast light micrograph of an adhered monolayer of epithelial-like cells. Dark-coloured granules/vesicles appear refractile under phase-contrast. **(B)** TEM micrograph of a CHSE-214 monolayer with adhered cells. Nuclei are large and often lobulated. Large secondary lysosomes are present within many cells. **(C)** TEM micrograph of control CHSE-214 cells to depict the secondary lysosomes and large, electron-dense vesicles/granules commonly seen within cells. Their lobulated nucleus is especially apparent in the cell on the right.

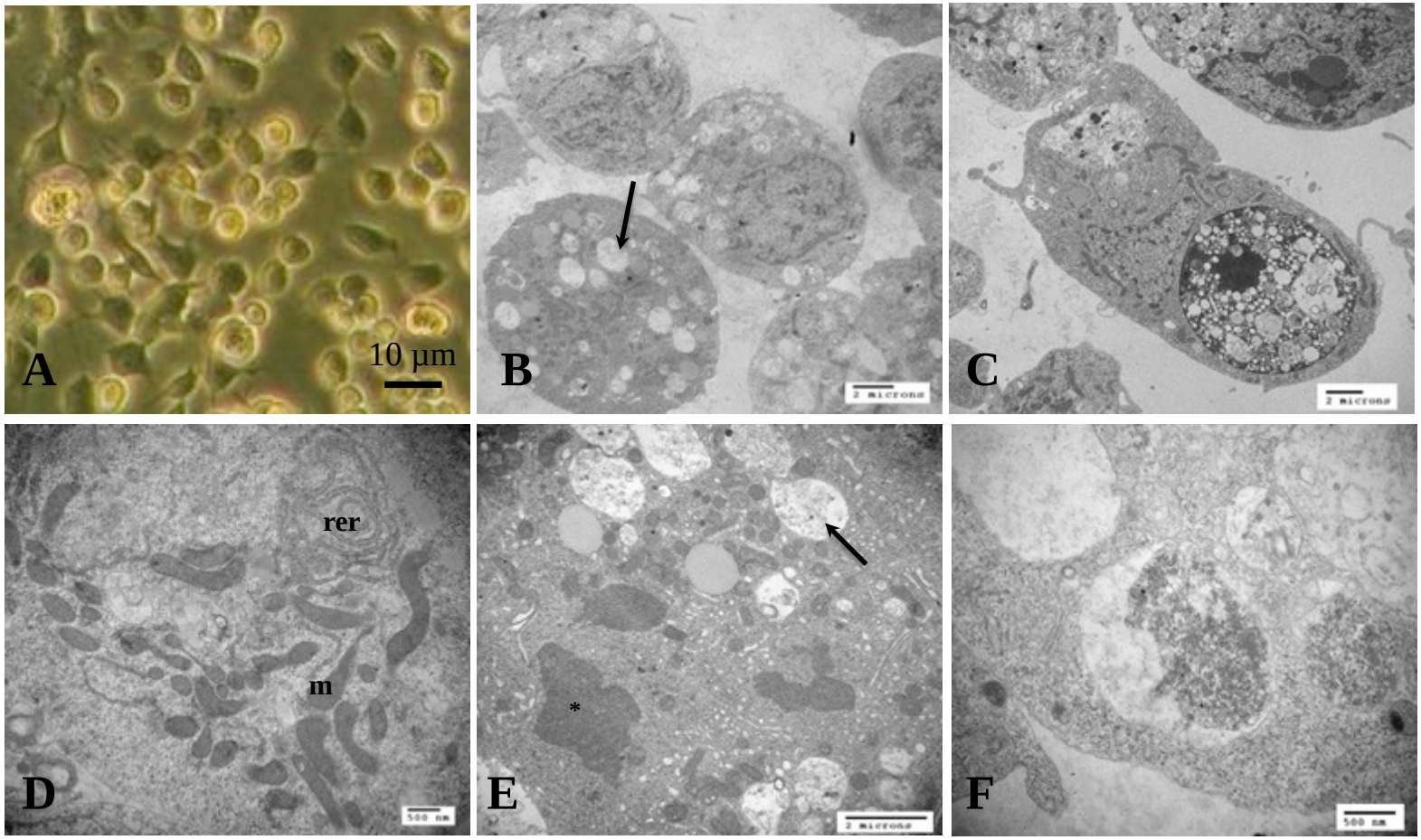
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Figure 5.3 (A-F): Control mosquito *Aedes albopictus* cells (AED) cells, 2 weeks post-plating. **(A)** Phase contrast light micrograph of semi-adherent cells within $L15^{+}$ media. Many cells appeared to contain refractile vacuoles. **(B)** TEM micrograph of AED cells in suspension. Many cells appear to contain electron-lucent vacuoles/vesicles (arrow). **(C)** TEM micrograph of an AED cell containing a phagocytized apoptotic cell. AED cells appeared to rapidly phagocytize any material suspended within the media. **(D)** TEM micrograph of the appearance of mitochondria (m) and rough endoplasmic reticulum (rer) in normal cells. **(E)** Image of the common components of an AED cell including electron-lucent vesicles (or vacuoles)(arrow that contain membranous and material. Also present are membrane-bound vesicles containing a more electron dense granular material (*). **(F)** Higher magnification of the fine granular material within the membrane-bound vesicles.

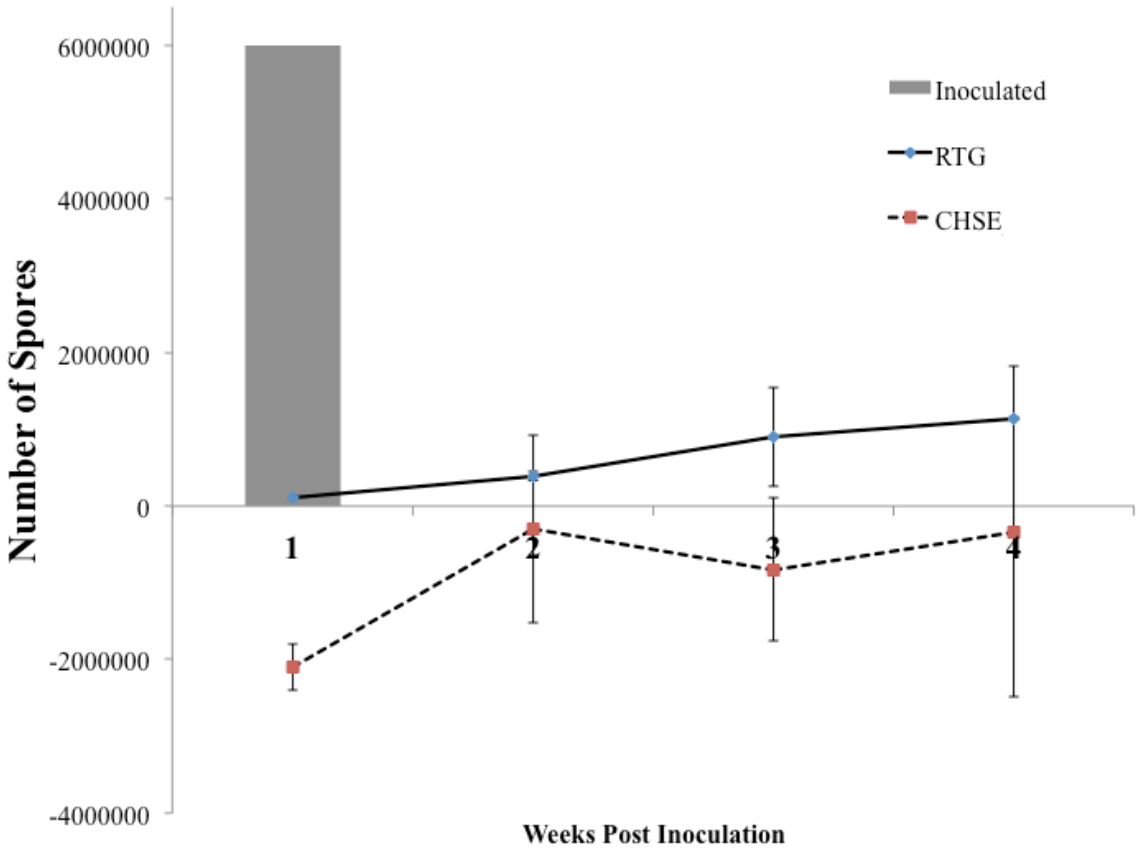

Figure 5.4: *L. salmonae* spore production over a 4 week period within rainbow trout gill epithelial (RTgill-W1) and Chinook salmon embryo cells (CHSE-214). Bar graph represents the inoculating dose ($6x10^6$ spores). Data are mean (±SE) spores numbers in each replicate flask (N=2 replicates).

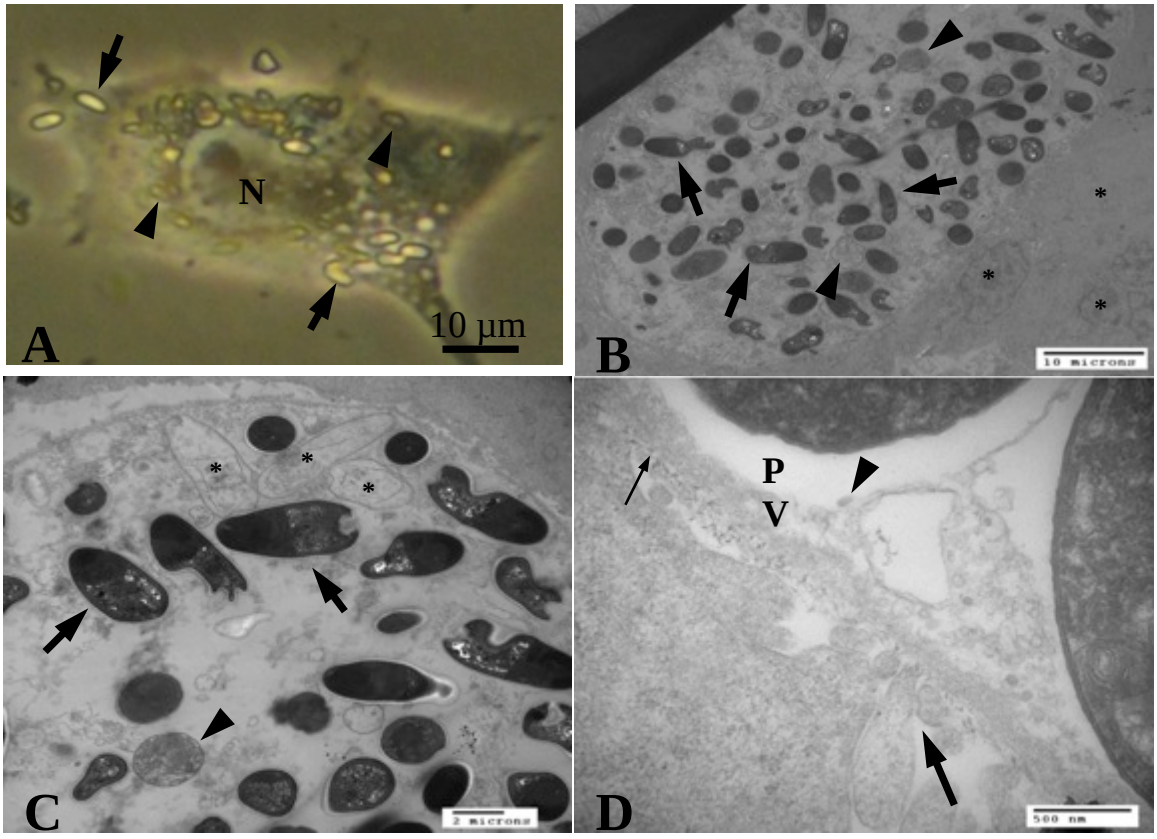

Figure 5.5 (A-D): Phase-contrast and TEM micrographs depicting a mature xenoma within RTgill-W1 cells containing *Loma salmonae* spores. (**A)** Phase-contrast micrograph of an adhered cell apparently filled with *L. salmonae* spores, 5d post-exposure (PE). Spores appear to be on the surface of (arrows), and within (arrowheads) the cytoplasm surrounding the nucleus (N) of the host cell. (**B)** TEM of a xenoma containing *L. salmonae* spores, 10d PE, surrounded by non infected cells (*). Many mature spores (arrows), and various degraded parasite developmental stages (arrowheads) are present throughout the cytoplasm of the xenoma. (**C)** Higher magnification of the same xenoma showing empty spores (*) along with mature spores (arrows) and degraded developmental stages (arrowheads) within the cytoplasm and the distinct cell membrane (small arrow) adjacent to surrounding non infected cells. (**D)** TEM showing blebbing (arrow) of portions of the peripheral cytoplasm and surrounding plasma membrane of the xenoma. Tubular elements (arrowhead) are evident within the parasitophorous vacuole (PV) containing mature *L. salmonae* spores.

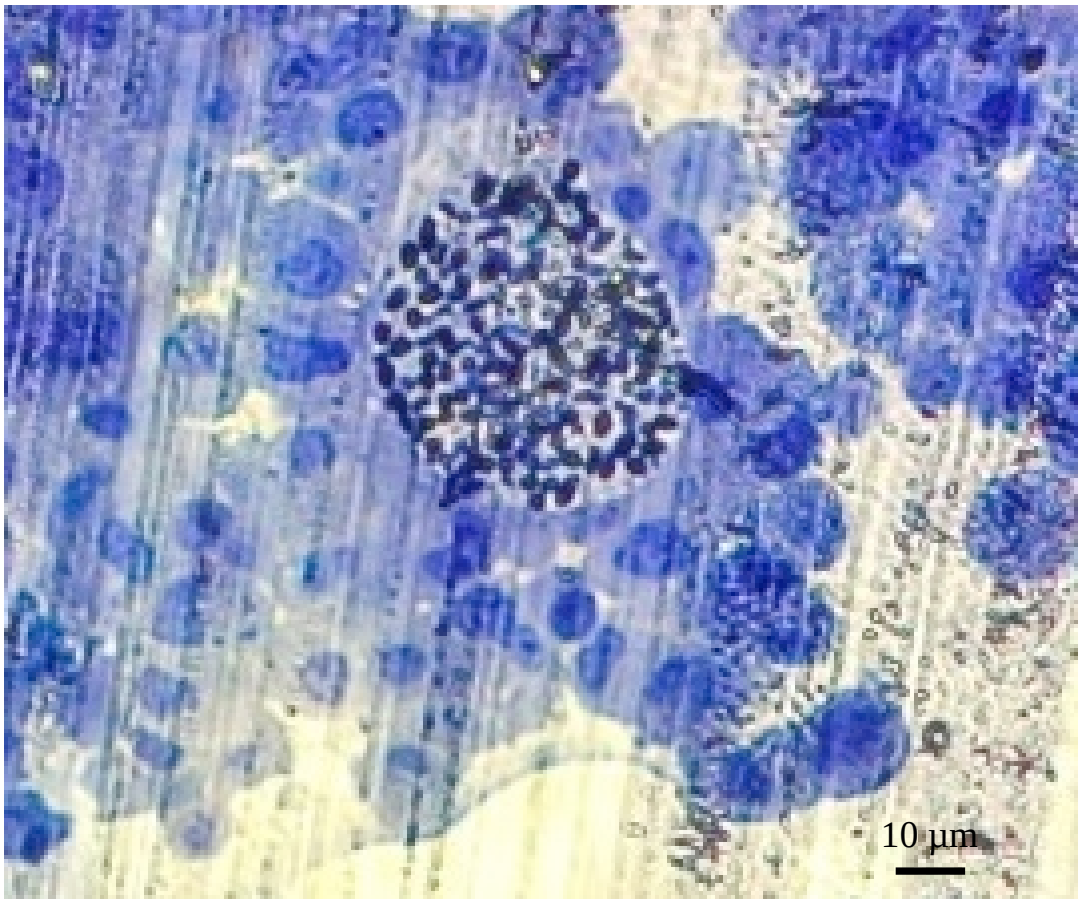

Figure 5.6: Light micrograph of a monolayer RTgill-W1 cells processed for TEM, 10d PE. The thick section was stained with toluidine blue. *L. salmonae* spores appear to be contained within the confines of one hypertrophic cell approximately 4x the diameter of the surrounding cells.

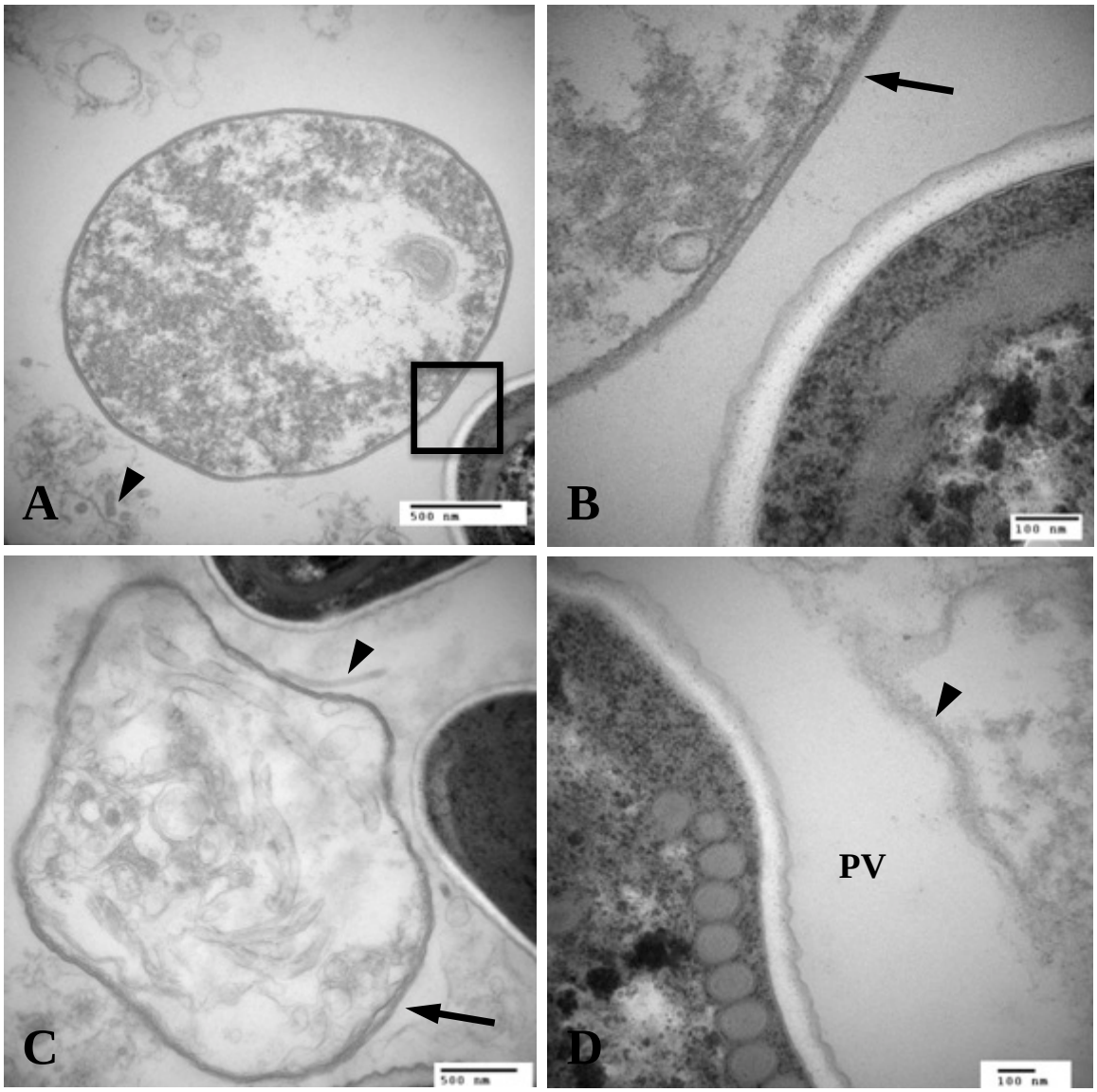

Figure 5.7 (A-D): TEM micrographs showing details of structures from the *L. salmonae* xenoma from Figure 5.5 (B-D). **(A)** A degraded developmental stage within a parasitophorous vacuole (PV). Tubular elements (arrowhead) are also present. **(B)** Higher magnification of the boxed-in region of (A) showing that the membrane of the degraded structure (arrow) appears to be from a developmental stage, possibly a sporont given the electron-density of the membrane. (C) Micrograph of tubular structures within the PV, and the presence of another degraded developmental stage of the parasite (arrow) that has an electron-dense membrane and contains disorganized tubular elements . (D) A portion of the parasitophorous vacuole showing the vacuole membrane (PV) and part of a mature, intact spore contained within the vacuole.

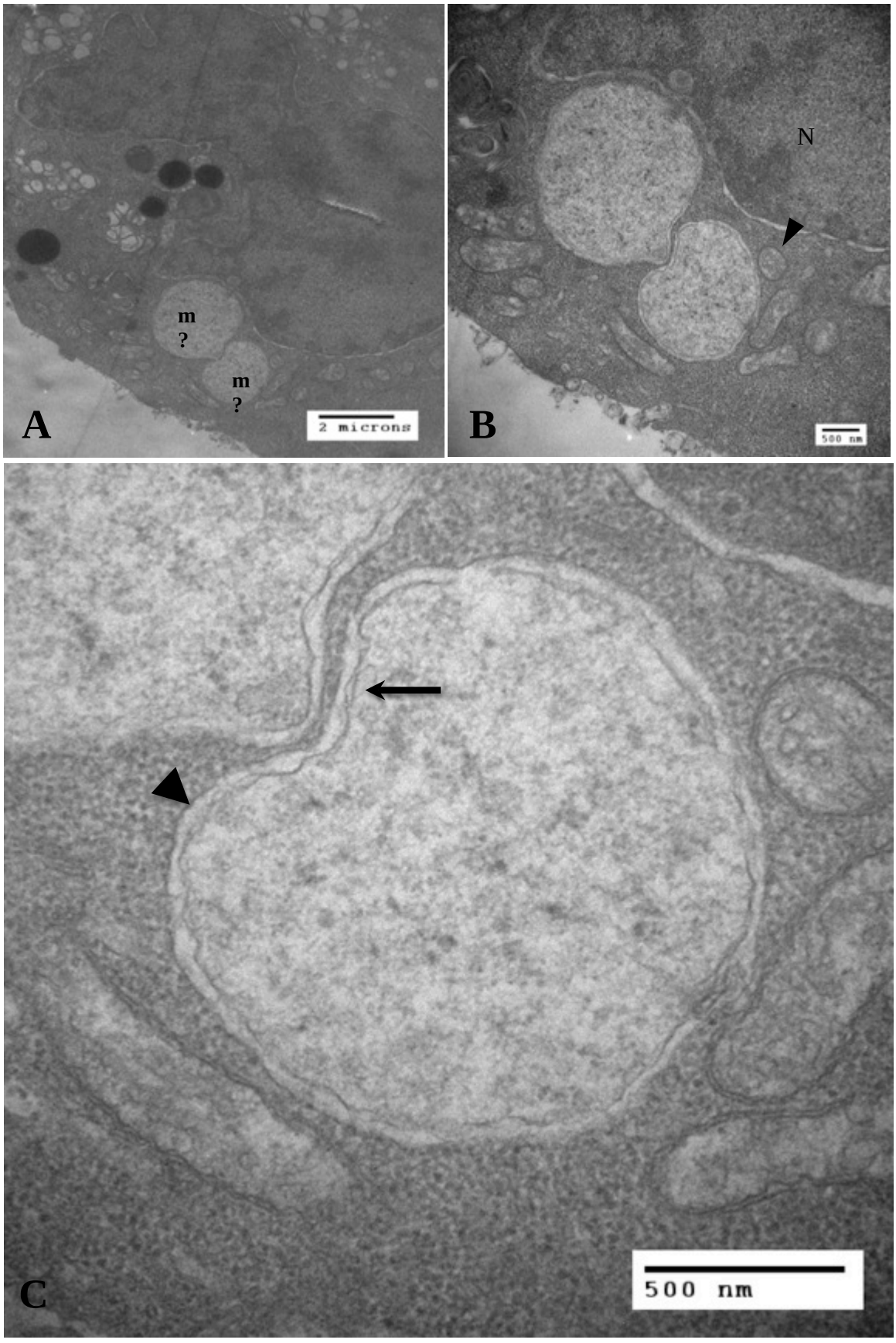
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Figure 5.8 (A-C): TEM micrographs of RTgill-W1 cells exposed to *L. salmonae* spores for 5 days. **(A)** Suspected meronts (m?) developing within the cytoplasm of a cell. The amorphous, electron-lucent material appears to be closely surrounded by a double membrane and sits within a membrane bound vesicle. Several host cell mitochondria are surrounding the vesicles. **(B)** A higher magnification of the meront-like structures, the surrounding mitochondria (arrowhead) and association with the nucleus (N) and cytoplasm. **(C)** High magnification of the meront-like structure clearly shows the amorphous, electron lucent cytoplasm surrounded by a double membrane (arrows). The entire structure sits within a membrane-bound vesicle (arrowhead)

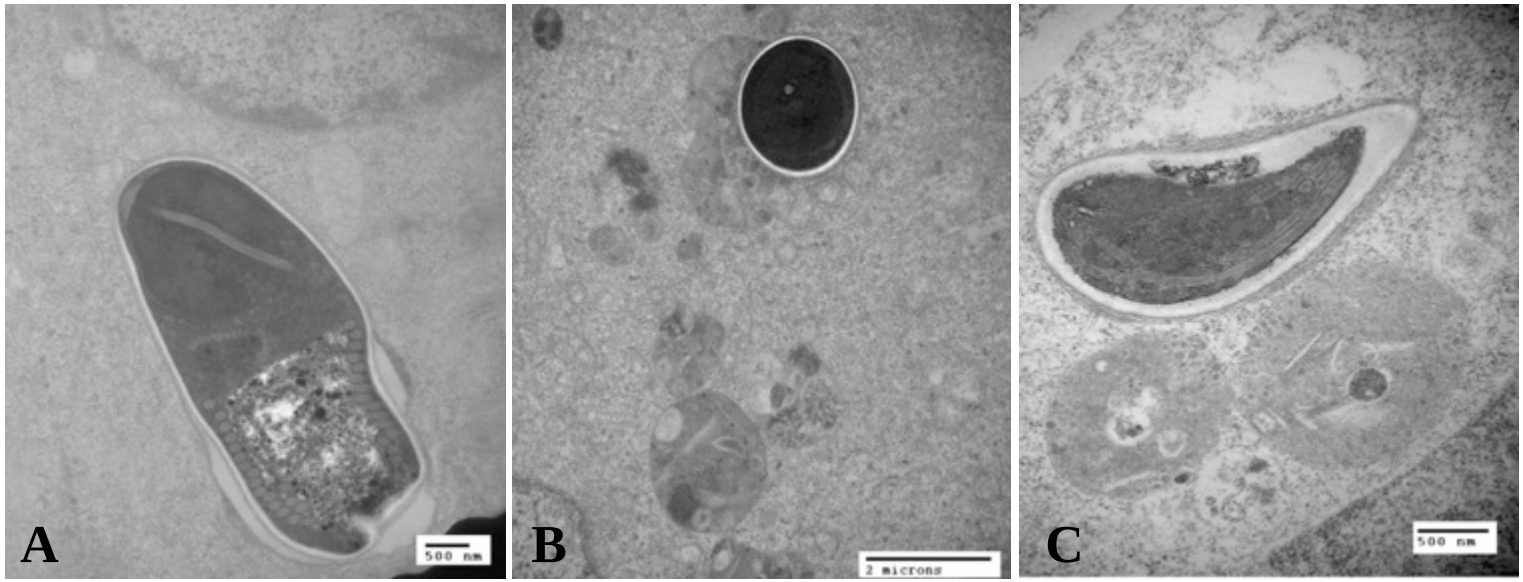

Figure 5.9 (A-C): TEM micrographs of RTgill-W1 cells and *L. salmonae* spores, 10 days post-exposure. It appears that spores are readily phagocytised by cells and digested within secondary lysosomes. **(A)** An intact spore within a membrane-bound vesicle within the cytoplasm. **(B)** An intact spore within a secondary lysosome within a cell. **(C)** A degraded spore within a secondary lysosome. Spores eventually degraded within the secondary lysosomes.

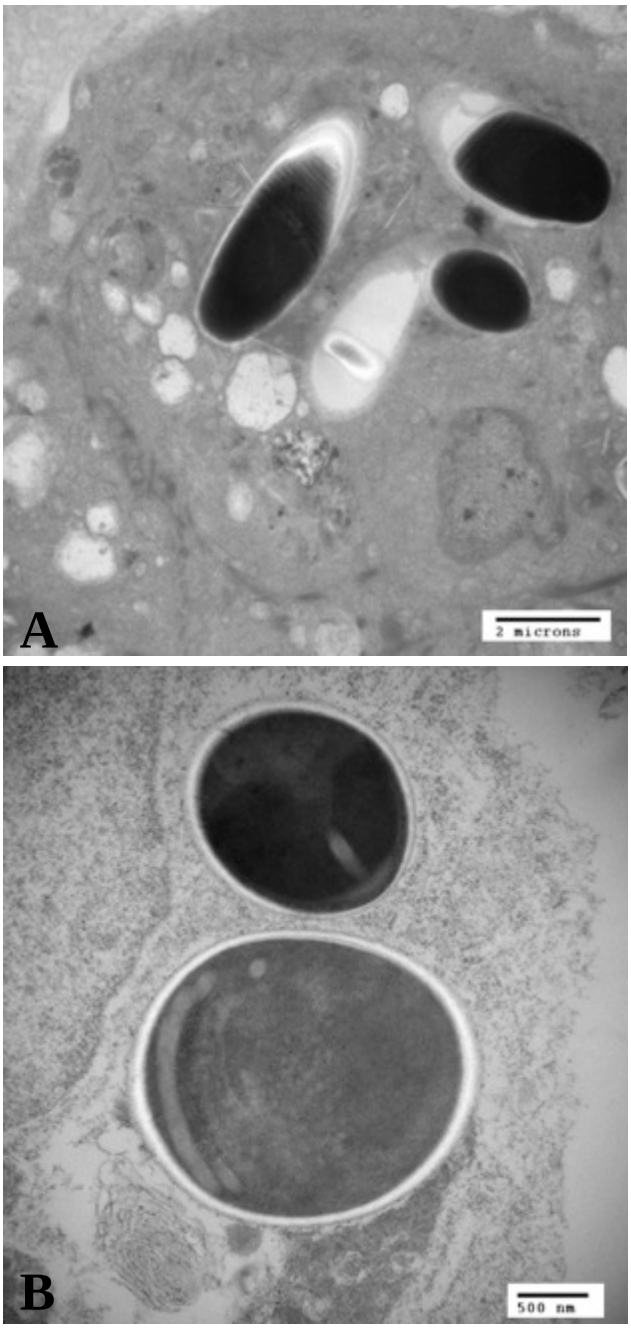

Figure 5.10 (A, B): TEM micrographs of CHSE-214 cells that have been exposed to *L. salmonae* spores for 10 days (A) and 2 weeks (B). **(A)** Cells readily phagocytized spores into phagosomes. Several cells were observed with multiple spores within their cytoplasm. **(B)** Spores were often seen in various stages of degeneration within secondary lysosomes.

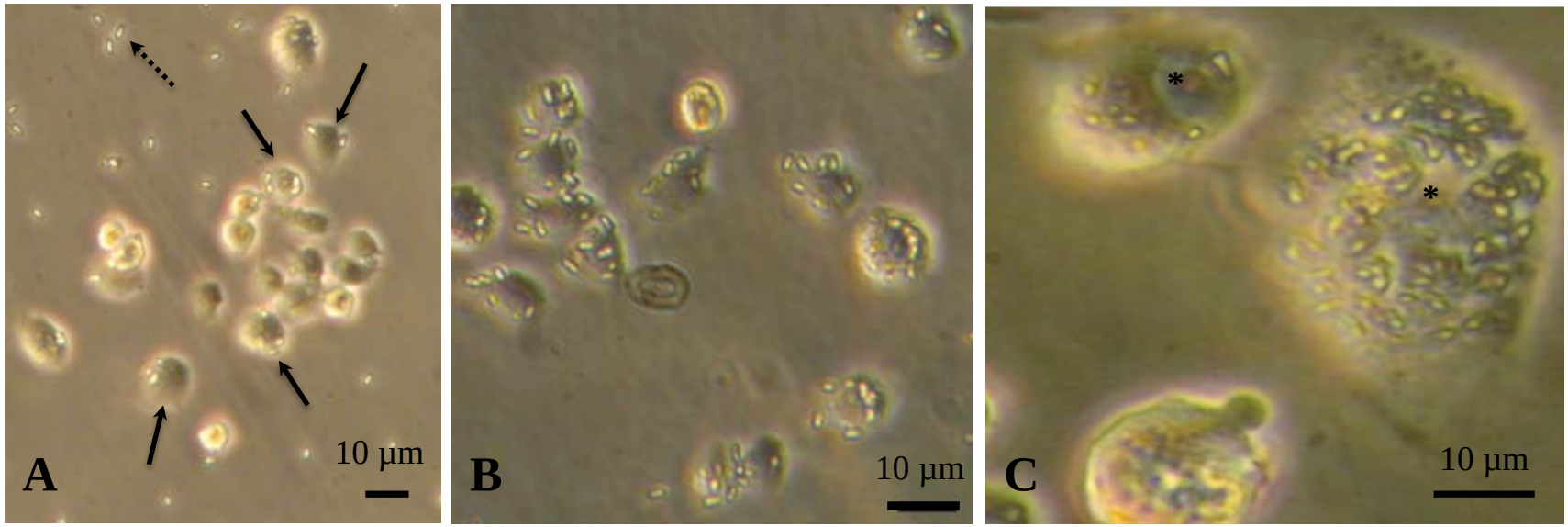

Figure 5.11 (A-C): Phase-contrast light micrographs of AED cells exposed to *L. salmonae* spores at a ratio of 10:1 (spores:cells) for 3 days. Spores readily become associated with the exterior of cells and shortly appear to be within the confines of AED cell membranes. **(A)** On day 1, *L. salmonae* spores (dashed arrow) appear as refractile, ovoid particles within the media. In some instances, spores appear to be closely associated (solid arrow) and possibly adhered to AED cells. **(B)** By 2 days, most spores appear associated with cells. **(C)** By day 3, many cells appear enlarged and filled with spores (*). Very few spores are free within the media by this stage.

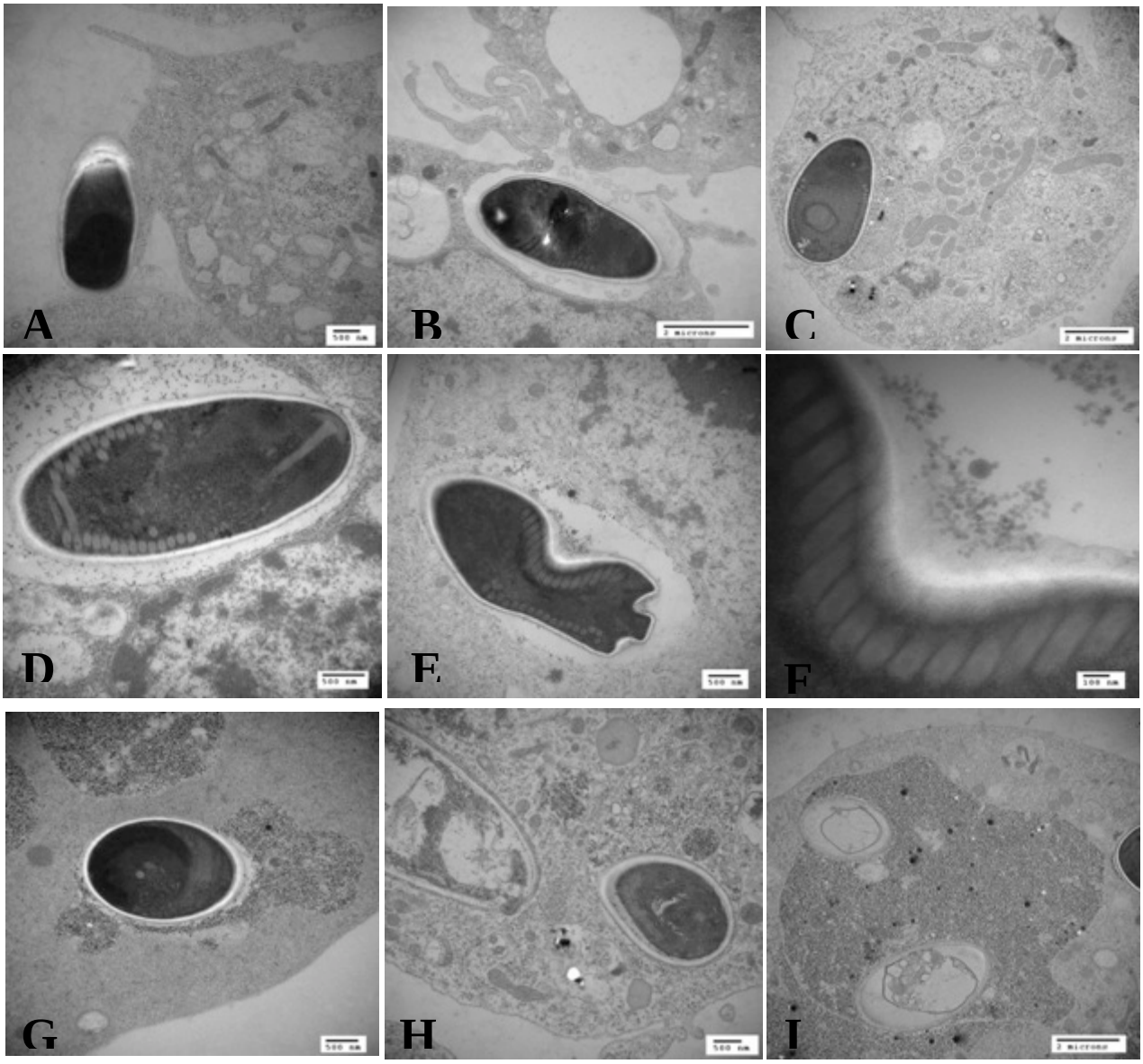
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Figure 5.12 (A-I). AED cells exposed to *L. salmonae* spores for 10 days and 2 weeks. TEM micrographs revealed that spores were being actively phagocytized and eventually degraded within secondary lysosomes. **(A)** An intact spore is apparently adhered to a pseudopod of an AED cell that appears to be beginning to phagocytize the spore. **(B)** Intact spore that appears to be entering a phagosome of an AED cell. The cell membrane is readily apparent, and there appears to be projecting pseudopodia surrounding the spore, forming a phagosome. **(C)** An intact spore is present within an AED cell. The spore is within a membrane-bound vesicle that is likely a secondary lysosome. The lysosome contains dense, electron-dense amorphous material. **(D)** An intact spore whose polar tube coils and polar disc are easily observable. The spore is within a membrane-bound vesicle with sparse, amorphous, electron-dense granules. **(E)** An irregularly shaped, intact spore within a membrane-bound vesicle within an AED cell. The amorphous granules appear to be adhered to the exospore. **(F)** Higher magnification of the portion of the spore whose exospore is closely associated with the amorphous granular material present in the membrane-bound vesicle. **(G)** An intact spore associated with a membrane-bound vesicle containing dense, electron-dense granular material. It is likely that the vesicle is a secondary lysosome. **(H)** Degraded spores within secondary lysosomes in AED cells. The spores have become disorganized and have lost electron density. **(I)** Degraded spores within a secondary lysosome.

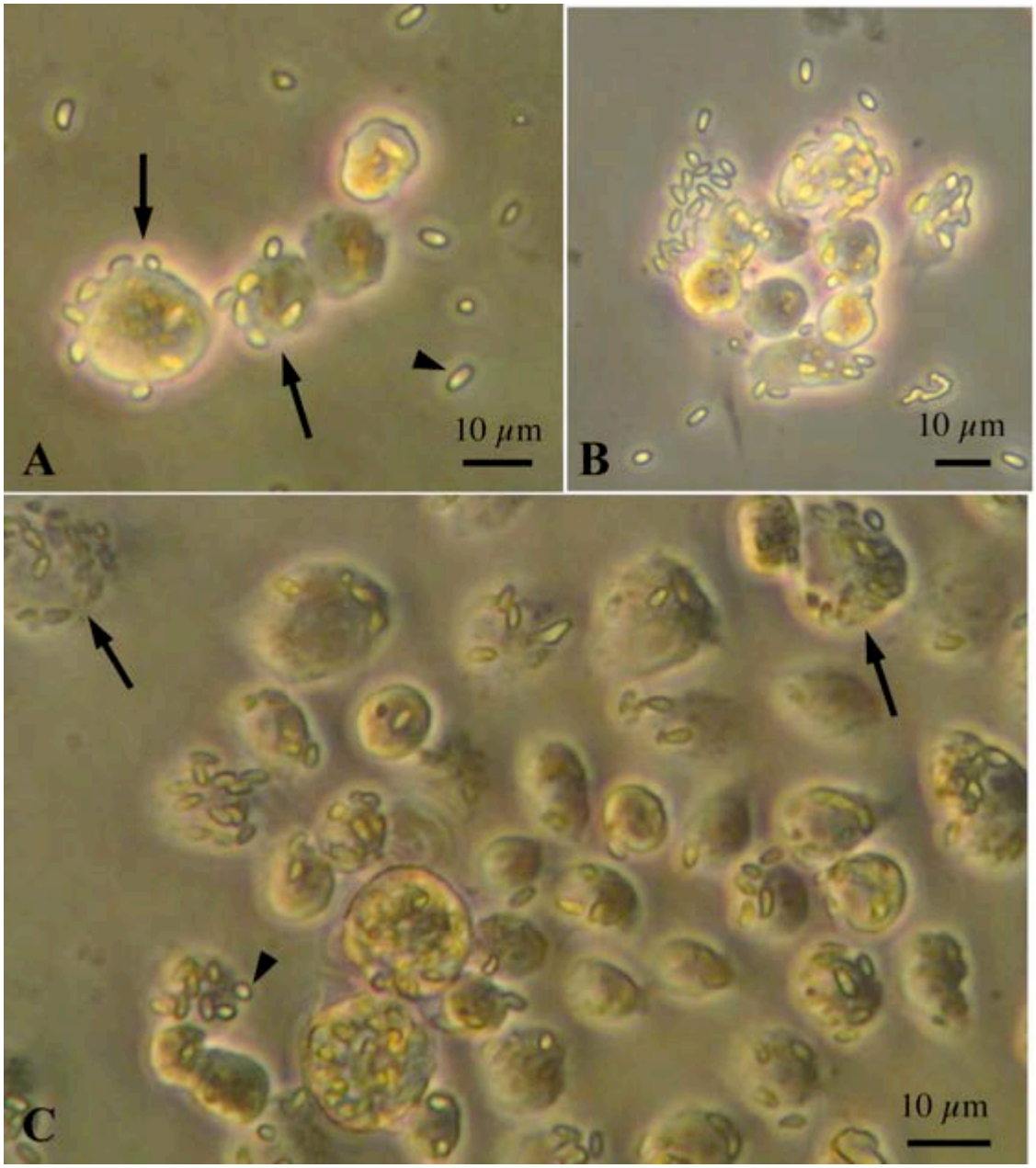

Figure 5.13 (A-C): Phase-contrast light micrographs depicting the association of *L. salmonae spores* and AED cells, shortly after exposure. **(A)** Spores and cells 1 hour after exposure. Phase-bright spores can be seen in the media (arrowhead) and closely associated with the surface of cells (arrow). **(B)** Spores and cells 2 hours after exposure. Phase-bright spores can still be seen in the media. Many more spores are associated with the surface of AED cells. **(C)** Spores and cells 24 hours after exposure. Phase-bright spores can still be seen in the media, and closely associated with cells. At this point, many cells appear to be full of phase-dark spores.

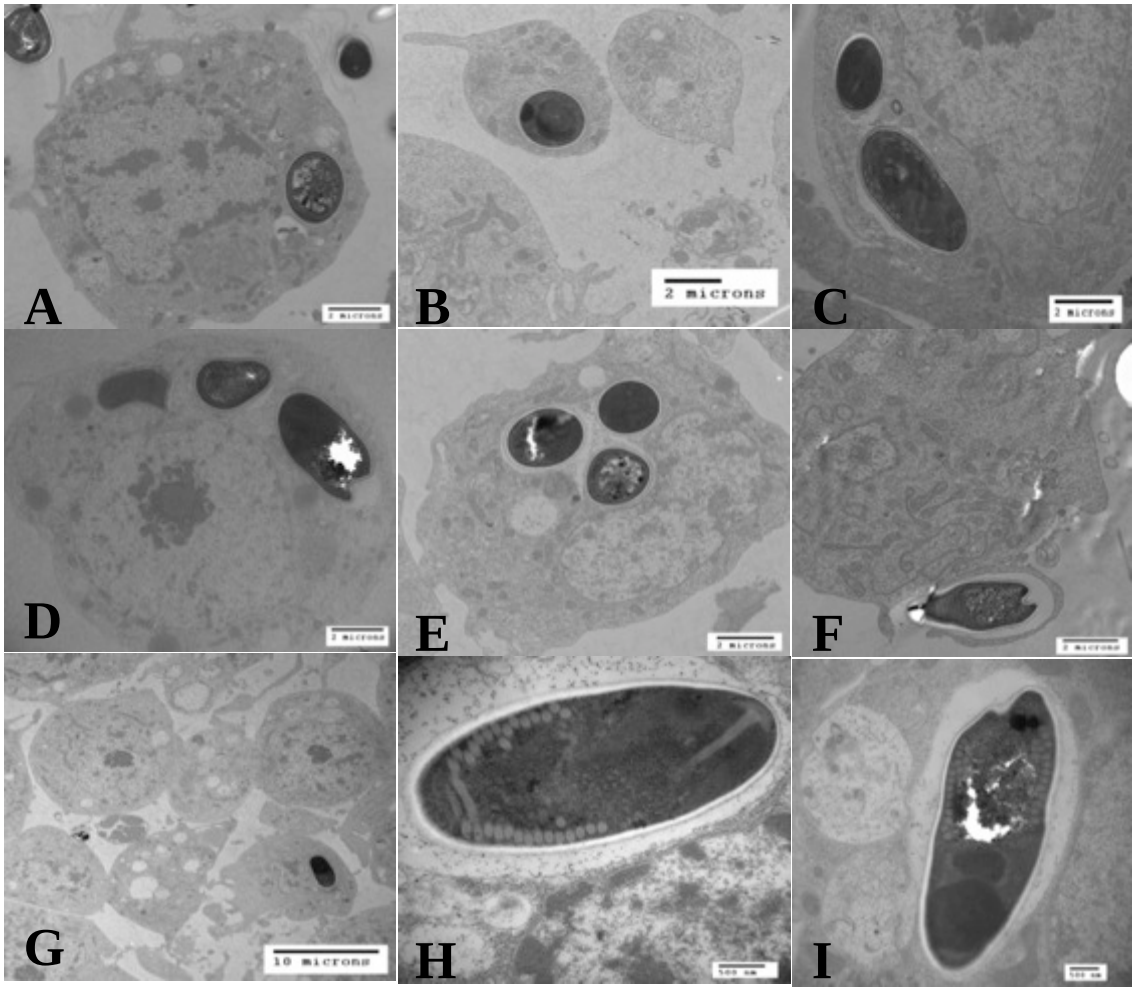

Figure 5.14 (A-I): TEM micrographs of AED cells exposed to *L. salmonae* spores at a ratio of 10:1 (spores:cells). TEM samples were taken at 6 hours post-exposure and then every day for 5 days, and then weekly for up to 3 weeks. Intact spores were evident at each time point. **(A)** AED cells and spores 6 hours post-exposure. Intact spores are free in the media, but are also seen within cells indicating that cells rapidly phagocytize the spores. **(B)** Intact spores seen within cells 1 day post-exposure. **(C)** Intact spores seen within cells 2 days post-exposure. **(D)** Intact and degraded spores seen within an AED cell 3 days post-exposure. It is unclear whether the spore was degraded prior to phagocytosis or after ingestion. **(E)** 3 intact spores seen within one membrane-bound vesicle in an AED cell, 4 days post-exposure. **(F)** An intact spore being phagocytized within an AED cell, 5 days post-exposure**. (G)** Intact spore seen within cells 7 days post-exposure. **(H)** Intact spore seen within a membrane-bound vesicle, 7 days post-exposure. **(I)** Intact spore seen within a membrane-bound vesicle, 3 weeks post-exposure.

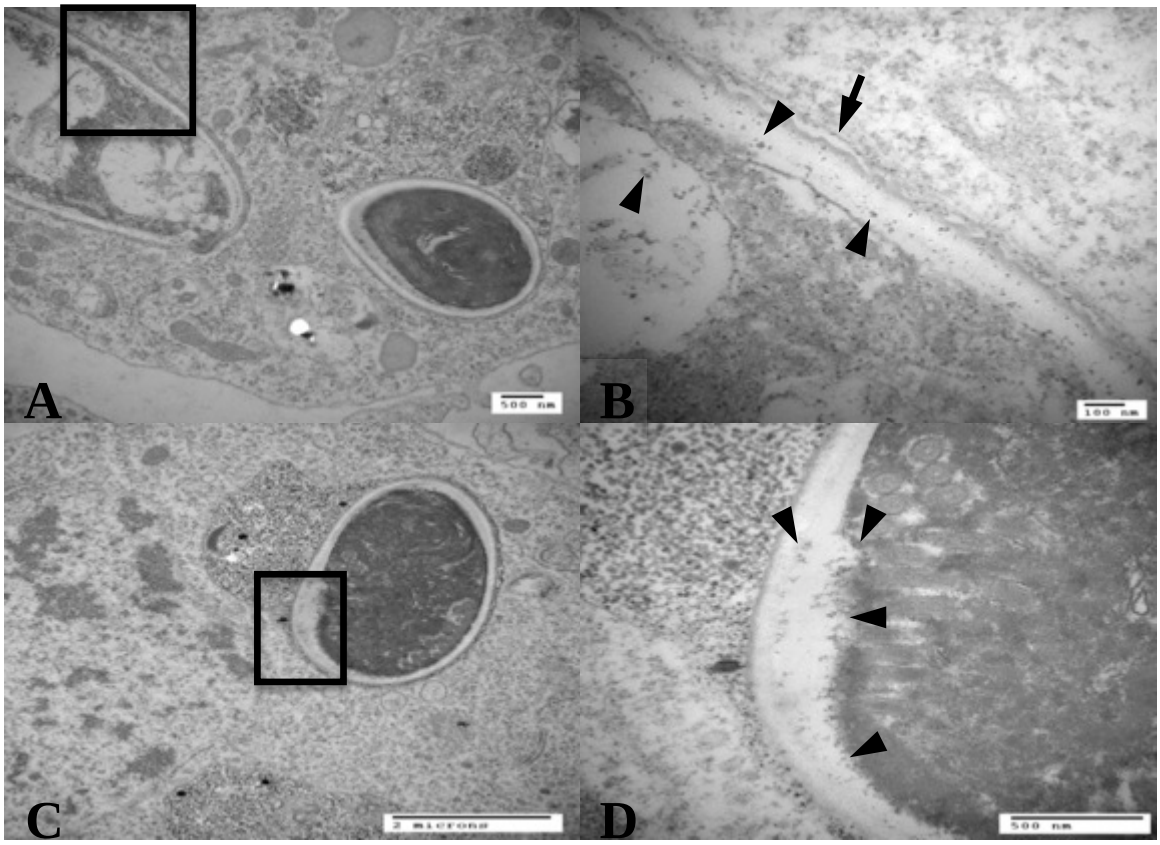

Figure 5.15 (A-D): Evidence of *L. salmonae* spore degradation within membrane-bound vesicles in AED cells, 10d PE. **(A)** Image of 2 degraded spores within membrane-bound vesicles in an AED cell. **(B)** Higher magnification of the outlined section from (A) of a spore within a membrane-bound (arrow) vesicle showing the electron-dense granular material (arrowheads) within the degraded spore. **(C)** Degraded spore within a membrane-bound vesicle densely filled with electron-dense granules. **(D)** Higher magnification of the outlined area in (C). The granular material appears to be aligned with the degrading surface of the spore (arrowheads).

5.5 Discussion

This study is the first example of microsporidian xenoma development within cell culture, verified by ultrastructural examination. Observations of a complete life cycle of *L. salmonae* within cell culture suggest that there is a potential to produce this parasite *in vitro*, using RTgill-W1 cells. Spore propagation was confirmed, albeit at low numbers. Further manipulations of this model are underway to determine if this type of spore development can be optimized. Neither CHSE-214 nor AED cells supported the development of *L. salmonae*. Spore numbers were difficult to quantify after exposure to AED cells, which rapidly phagocytized spores and other material. Since freezing and cryopreservation following standard methods do not preserve the viability of *L. salmonae* spores, AED cells were explored as a component of cryoprotection. Preliminary evidence suggests that spores frozen within cells maintained some *in vivo* viability, 6 months after storage.

Interestingly, the xenoma observed at 10 days PE was mature and contained degraded developmental stages. Within fish, *L. salmonae* xenomas do not mature until ~6 weeks PE (Speare & Lovy, 2012). Observations of hypertrophic, spore-filled cells by 3 days PE, and the mature state of the xenoma at 10 days PE suggest that the development of the xenoma occurs in a short period compared to development *in vivo*. Regardless, the length of the developmental period *in vivo* may reflect the selective xenoma development within gill tissue. *In vivo* xenoma production in the gills is biphasic, and although the majority of xenomas form around weeks 5-6 PE, there are others that form as early as 3 weeks PE (Speare et al., 1998). *L. salmonae* circulates within the body of fish *in vivo* for 2 weeks prior to being detected in the gill by *in situ* hybridization, and thus mature xenomas by 3 weeks would suggest a 1 week maturation

period, consistent with our observations (Speare et al., 1998). Sub-optimal culture conditions may have contributed to early degradation (*e.g.,* nutrient availability).

Further *in vivo* and TEM studies revealed suspected meronts within RTgill-W1 cells 5 days PE. The identity of the structures was not verified; future studies involving immunohistochemistry could help identify developmental stages. The MAb developed for *L. salmonae* appears to be specific to components of the mature spore wall and thus would not be suitable for immunohistochemistry analysis of early developmental stages. It would be ideal to develop a meront-specific MAb to observe developing stages under light microscopy and TEM. For example, meront specific MAbs have been developed for human-infecting microsporidians (*e.g.*, *E. cuniculi*; Fasshauer et al., 2005). For another example, ultrastructural *in situ* hybridization can detect nucleic acid sequences at the ultrastructural level (Le Guellec, 1998). Similarly, a DNA-stain, such as DAPI (4',6-diamidino-2-phenylindole), could be useful *in vitro* in combination with Calcofluor white (CW). DAPI would stain parasite DNA and CW the chitin endospore wall (Harrington & Hageage, 2003). The combination of stains would differentiate meronts from life stages that contain the chitin endospore (Monaghan, 2011). Ongoing studies using an optimized model may also help capture more numerous xenomas with varied developmental stages for ease of comparison.

Only low-levels of spore production were seen on RTgill-W1 cells. No parasite development was observed in CHSE-214 or AED cells. Spore numbers in CHSE-214 flasks decreased, rather than increase, as they did for RTgill-W1 cells. The presence of degraded spores within secondary lysosomes suggest that both cell lines were able to phagocytize and digest *L. salmonae* spores. Perhaps the sporoplasm is the only portion of the spore that can block fusion of the phagosome with the lysosome to allow

development. However, after exposure to the low pH environment of the phagolysosome, germination may have been triggered which may account for the increase in spore numbers observed over the 4 week period.

Development of the parasite was not optimized, perhaps for several reasons. First, spores were exposed to cells adhered on a monolayer. Suspended spores within the media may not have reached cells during polar tube eversion. Second, the conditions within the media may have been inappropriate for germination. Many of the cells free in the media, and those within secondary lysosomes were intact, suggesting that this was a likely contributing factor. Third, the nutritional components of the media may not have been optimal for parasite development. In order for intracellular proliferation and spore maturation to occur, microsporidia rely heavily on the metabolic output of the host cell (Cuomo et al., 2012). Microsporidians upregulate host-cell genes responsible for nutritional and metabolic pathways to support their growth (Holt et al., 2013). Further investigation into factors that promote germination and metabolic maintenance could help optimize the model.

Several strategies address the above issues. Finding a media that spores reliably germinate in, that is also not toxic to the host cells would be ideal. In fact, AED cells could be reevaluated as potential host cells if they phagocytize sporoplasms, which is another mode of infection that microsporidia utilize (Dunn & Smith, 2001). *L. salmonae* initially infects intestinal cells, and the pH shift from the acidic stomach to the alkaline upper intestine (via pancreatic enzymes) likely triggers germination. Manipulating the pH of media, or adding digestive enzymes in the presence of both freely suspended spores and cells may be useful. Evidence also suggests that spores can be primed to adhere to cells in culture using chemicals such as $MgCl_2$ (McLeod, 2012). Additional

nutrients could also be added to the media to support the increased metabolic needs of host cells supporting developing parasites. Microsporidia appear to alter cell functioning in a similar way to cancer cells to support rapid growth (Fan et al., 2012). Usually cancer cells increase their rate of anaerobic glycolysis; thus nutrients and enzymes that support glycolysis could be added to the media (Vander Heiden et al., 2009). However, microsporidians apparently rely on host-cell mitochondria for oxidative phosphorylation (OP); nutrients that support OP could also be tested. Media that support the growth of cancer cell lines may be useful in this model. Temperature and pH also affect metabolic rates of cultured cells (Ozturk & Hu, 2005) so these parameters must also be optimized, especially if pH will be manipulated to stimulate germination. Cell health can be monitored with Trypan blue and spore germination using phase contrast microscopy (Monaghan, 2011).

Microsporidian xenomas have not previously been shown to develop within cultured cells. One study of a human microsporidian did elicit some xenoma-like growth within E6 cells (Leitch et al., 2005). However, the growth was determined not to be true xenomas; they were aggregates of multiple E6 cells fusing together to form large cells with many developing spores within them (Leitch et al., 2005). Lores and colleagues (2003) elicited development of fish-infecting *Glugea* sp. spores within the mosquito *Aedes albopictus* cell line without true xenoma formation. More recently, development of *L. morhua* within a larval cod-derived cell line (GML-5) was shown to occur slowly (~36 days PE) and at extremely low infection levels; xenoma development was not confirmed or discussed (McLeod, 2012). *L. morhua* specifically infects Atlantic cod (*Gadus morhua*) and failed to develop within haddock embryo (HEW) and RTgill-W1 cells, further suggesting that host specificity is extremely important in xenoma-forming

microsporidians (McLeod, 2012). RTgill-W1 cells appear to be permissive for xenoma development by *L. salmonae*, which is intuitive since the major site of infection in salmonids is within gill tissue in endothelial, and other epithelial-type cells (Speare & Lovy, 2012).

Apparently xenoma formation can occur in cell culture without influence from the host organism. With modifications, the *L. salmonae*-RTgill-W1 cell model should be useful for further study and propagation of *L. salmonae* and other xenoma-forming microsporidians. Further studies probing how, when, and why xenoma development occurs may be possible using our model. It may even be possible to determine the specific inducing factor(s) that elicit hypertrophic growth and early development in microsporidian xenomas (Lom & Dyková, 2005). Further development and optimization of an *in vitro* technique supporting the entire life cycle of this microsporidian will also provide, when compared to the current *in vivo* model, a more functional platform for broad and in depth assessment of host-pathogen relationships at the cellular level.

AED cells appeared to be continuously endocytosing fluid from the media, as evidenced by the presence of many vacuole-like structures in TEM and light micrographs. Spores appeared to easily adhere to AED cells, perhaps stimulating rapid phagocytosis. The non-adherent nature of AED cells could also make them more effective at contacting spores in suspended media. This cell-spore association was not observed in the other 2 cell lines. However, no evidence of spore development was seen in AED cells, despite how effectively the cells phagocytized the spores. Additionally, most spores seen within AED cells were still intact, and appeared to degrade at a slow rate. Since for TEM micrographs, uranyl acetate stains protein-rich lysosomes with high

contrast, spores within the electron-lucent vacuoles are likely recently formed phagosomes (Hayat, 2012). Spores within the more electron-dense phagolysosomes occurred less frequently, suggesting that fusion occurred less commonly than exocytosis of intact spores. Many intact spores were seen entering phagosomes at all stages of the experiment, suggesting that spores were rapidly being taken up, and perhaps eliminated/exocytosed by cells.

AED cells could potentially be utilized as a component of a cryoprotectant for *L. salmonae* spores. The gelatin trial showed promise with almost 50% of fish becoming infected after consuming spore-laden AED cells in gelatin. Infection rates were similar to purified spores being processed into gelatin and fed to fish. Perhaps if the spore dosage was increased, this could have yielded better results. Validating this experimental result may help improve the current disease model. The cryopreservation of *L. salmonae* spores will reduce the demand for experimentally infected rainbow trout needed for maintenance of this parasite using the disease model outlined by Speare and colleagues (1998). Successful cryopreservation will also provide researchers with a consistent isolate or strain of spores for a variety of analyses.

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Chapter 6.0 General Discussion

6.1 Conclusions and Discussion

Integrated multitrophic aquaculture (IMTA) has the potential to mitigate concerns related to traditional monoculture (*e.g.,* environmental effects such as nutrient loading). Utilizing an ecological approach to nutrient management may create a more diverse industry that has fewer environmental impacts. However, integrated culture systems have the potential to harbor pathogens that thrive in complex environments. This thesis has explored how an environmentally resistant microsporidial pathogen, *Loma salmonae,* can be used as a model in determining how environmentally stable pathogens behave within an IMTA setting. The work presented in this thesis has covered three main themes - all utilizing manipulations of the *L. salmonae*-rainbow trout (*Oncorhynchus mykiss*; LS-RT) disease model. First, blue mussels (*Mytilus edulis*) were added to the disease model to elucidate the role of environmentally resistant pathogens in complex IMTA settings. Next, *in vitro* spore viability was correlated to *in vivo* viability using a *L. salmonae* spore specific monoclonal antibody-propidium iodide (MAb-PI) dye exclusion assay. Lastly, studies were conducted working towards integrating cell culture into the traditional LS-RT disease model.

The established LS-RT disease model was manipulated to include blue mussels as a representative of a filtering bivalve likely to be utilized in IMTA systems. In order to identify *L. salmonae* spores within mussel tissues and excrement, a spore-specific MAb assay was utilized. The assay proved useful in determining the membrane-integrity of spores by adding a PI dye exclusion test. Large numbers of spores were required for many of the experiments outlined in the present thesis, which led to an investigation in the propagation of viable *L. salmonae* spores using cell culture techniques.

IMTA systems not only have the potential to mitigate nutrient loading issues, they may help control important fish pathogens that thrive in monoculture. For example, filtering bivalves have been shown to ingest and deactivate problematic parasites (*e.g.*, *Lepeophtheirus salmonis*; Bartsch et al., 2013), viruses (*e.g.*, Infectious Salmon Anemia Virus; Molloy et al., 2014), and bacteria (*e.g.*, *Renibacterium salmoninarum*; Paclibre et al., 1994). However, the above pathogens are relatively environmentally fragile with low stability and host diversity. It was theorized that a pathogen with an environmentally resistant life stage, such as *L. salmonae* spores, would remain viable in the presence of filtering bivalves. Indeed, data from Chapter 2 verified that *L. salmonae* spores remain infective following exposure to actively filtering blue mussels. The viscera from exposed mussels were able to transmit infection to naïve rainbow trout at least 28 days following exposure to spores, and this period of infectivity was not affected by the water temperature at which the mussels were housed (Chapter 2). The findings from this study are intuitive as mussels, and other filtering bivalves, are commonly used as bioindicators for the presence of important environmentally stable human-associated pathogens (*e.g.*, *Toxoplasma gondii* oocysts, Arkush et al., 2003; *Cryptosporidium parvum* oocysts, Tamburrini & Pozio, 1999).

Results from Chapter 2 reveal that mussels filter and expel viable *L. salmonae* spores via feces and pseudofeces, which are then available to be consumed by fish. This result was further investigated in Chapter 3 where spores were exposed to, actively filtering, individual mussels, for a 4 hour period. Mussel feces and pseudofeces were sampled at regular intervals and processed to detect the presence of spores. It was determined that mussels filtered the majority of exposed spores, and most were expelled via the feces within 24 hours. However, mussels continued to expel spores at low levels

for up to 7 days post-exposure. The MAb-PI spore detection and viability assay outlined in Chapters 3 and 4 were utilized to determine spore presence and viability within mussel excrement. There was no observed change in membrane-integrity if detected spores. However, further study is needed to differentiate between viable and empty spores. The results from this study verify that mussels are unable to bioremediate *L. salmonae* spores and readily expel infectious spores into the environment while also retaining infective spores within their viscera for up to 28 days post-exposure. Therefore, in an IMTA setting where *L. salmonae* is a concern, mussels have the capacity to act as a disease reservoir.

Complex aquaculture systems, such as IMTA, will result in the emergence of pathogens that thrive in complex ecosystems. Studies, such as those outlined in Chapters 2 and 3 can help us forecast the types of diseases that may become problematic in an IMTA setting. There are four aspects of disease ecology that describe pathogens that would be successful in trophic-based aquaculture systems. First, pathogens that utilize intermediate hosts may emerge in an IMTA setting, especially metazoans such as digenean trematodes that use molluscs and invertebrates as intermediate and paratenic hosts. Additionally, myxosporidia such as *Ceratomyxa shasta*, reside within their intermediate host in complex habitats, such as polychaete worms on freshwater mussels, before being exposed to their definitive host (Bartholemew et al., 1997). Second, which is related to the first, pathogens that can live in complex environments may be more problematic in an IMTA setting. In monoculture, biofouling is a regular problem that often has to be managed. However, when you add complexities such as mussels and seaweed, it would be difficult to manage the habitats and biofilms that these organisms support (*e.g.*, the intermediate host for *C. shasta* lives on mussels). For example,

Neoparamoeba peruans, the causative agent for amoebic gill disease, proliferate within biofilms on fin-fish netting (Tan et al., 2002), as do the bryozoans that carry the causative agent of proliferative kidney disease (*Tetracapsuloides bryosalmonae*; (Bruno et al., 2006; Morris, 2012). Third, pathogens that can infect multiple hosts would likely find multiple refuges within IMTA systems, such as infectious pancreatic necrosis virus (IPNV) and viral hemorrhagic septicemia virus (VHSV) (Gregory et al., 2007; Gomez-Casado et al., 2011). Lastly, which is relevant to the findings in this thesis, pathogens with environmentally stable life stages, and those that can withstand environmental variability will likely be problematic within IMTA systems. There are examples of environmentally stable viruses (*e.g.,* IPNV), bacteria (*e.g.*, Mycobacteria), metazoan and protozoan parasites (*e.g., L. salmonae*), all of which are environmentally stable or produce life stages that can withstand environmental variability (*i.e.*, cyst formation). Further knowledge on the complexities of disease ecology and the implementation of more complex aquaculture systems, adding several trophic levels will increase the complexity of disease dynamics within these systems. The results from Chapters 2 and 3 verify that spore-forming, environmentally stable pathogens would likely have more complex ecologies within IMTA systems that may lead to increased pathogen pressure.

The studies outlined in Chapters 2 and 3 required high spore numbers for experimentation. It was also necessary to determine a way to confidently identify *L. salmonae* spores within mussel excrement and to determine spore viability in a reliable way. These needs led to the modifications of the LS-RT disease model outlined in Chapters 4 and 5. Chapter 4 outlines the methodology used to demonstrate the use of a MAb-PI exclusion test to verify spore membrane-integrity *in vitro*. The studies outlined in Chapter 5 were designed to find a way to increase spore numbers without using large

numbers of fish for the model (*i.e.*, the concept of "reduction"), along with the possibility in reducing the time required to obtain spores from the *in vivo* LS-RT model. With the prospect of producing large number of spores, the possibility of storing spores within liquid nitrogen for long-term storage of the disease model was explored.

The traditional LS-RT model assesses spore viability via the presence of xenomas on trout gill filaments following the six-week incubation period. This model is preferred as it observes the completion of the developmental cycle of the spores *in vivo*, which is of ultimate relevance when determining spore viability. However, it was important to develop a quick and simple way to assess the identity and viability of spores in suspension, especially when mixed with mussel excrement. Therefore, the aim of Chapter 4 was to correlate apparent spore viability, via the described *in vitro* assay, with spore infectivity *in vivo*. The viability assay developed in Chapter 4 utilized a known *L. salmonae* reactive MAb, combined with a PI dye exclusion assay. PI is impermeable to viable cell membranes, but will bind to nucleic acids within compromised cells and emit a red fluorescence that provides an excellent contrast to the green fluorescence of the MAb assay (Amigo et al., 1994; Stober et al., 2001). The MAb-PI *in vitro* assay results were concordant with *in vivo* results of spores that had been heat-inactivated. Additionally, limited research has been published on the effects of disinfectants on microspordian spores, especially for *L. salmonae* (John et al., 2005). Once the *in vitro* assay was verified, several chemical treatments were applied to isolated spores to determine their effectiveness at deactivating spores. Chemical treatments such as sodium hypochlorite, iodine, 70% ethanol and 1% Virkon appeared to be effective disinfectants, whereas formalin and hydrogen peroxide did not appear effective based on the *in vitro* assays. However, when compared to *in vivo* results,

sodium hypochlorite appeared to be the most effective treatment, especially given the high concentration of iodine required to deactivate spores (>300 ppm). This methodology will be useful for the management of *L. salmonae* within fish culture. If spores are present on a farm or in a hatchery, the above methods can help identify spores, and allow managers to quickly verify the success of disinfection. Additionally, the viability testing is useful in determining that spores are deactivated prior to being utilized as a whole-spore killed vaccine.

Microsporidians have been successfully cultured and propagated within cell culture systems for research and other purposes. In fact, the microsporidian *Paranosema locustae,* which is used as a biocontrol for locusts via hindering swarming behaviour, is commercially propagated using cell culture techniques (Khurad et al., 1991; Shi et al., 2014). To increase spore numbers using less fish and in a faster time frame, the culture of *L. salmonae* was attempted. Rainbow trout gill epithelial cells (RTgill-W1) were chosen as the gill is the most common host tissue infected in MGDS. Chinook salmon embryo cells (CHSE-214) were chosen as Chinook salmon (*Oncorhynchus tshawytscha*) are the most susceptible to MGDS infections. Lastly, Asian tiger mosquito (*Aedes albopictus;* AED) cells were chosen as they showed promise in supporting the growth of another fish-infecting microsporidian, *Glugea* spp. (Lores et al., 2003). RTgill-W1 supported the growth and development of *L. salmonae in vitro* (McConnachie et al., 2015) (Chapter 5). The technique used was the first study to show the development of xenomas within cell culture. Further studies are required to streamline and optimize the protocol. The results from this study represent an exciting opportunity to probe how microsporidians capitalize on cell physiology in order to support xenoma development (Chapter 5).

With the extra spores needed for the studies within this thesis, it was required that spores be stored for future usage. However, pilot studies replicating the storage techniques of spores outlined by the American Culture Type Collection (ACTC) were unsuccessful (Chapter 5). The method used yielded very low infection *in vivo*, and low viability numbers *in vitro*. During cell culture studies, it was observed that AED cells readily phagocytize spores with maintenance of *in vitro* membrane-integrity (within 24 hours). It was hypothesized that the AED cells could serve as a cryoprotectant for spores being frozen and stored. By utilizing the AED cells as a cryoprotectant, viability studies revealed low recovery after thawing, but viability should be high enough to cause infection *in vivo*. *In vivo* studies revealed low levels of infection, which was promising. It is advisable that the studies be repeated in order to optimize and verify results. The successful cryopreservation of spores would reduce the amount of experimentally infected animals needed for the routine maintenance of *L. salmonae* for the disease model. It would also allow the preservation of specific strains and isolates for future analysis and use. In theory, cryopreservation of fungal spores maintains strain genetics, should be relatively straightforward, and can maintain viability for up to 40 years (Ryan & Smith, 2004). However, liquid nitrogen levels must be maintained at all times or samples can be easily lost. Several species of microsporidia have been successfully cryopreserved, as have infected cell cultures (Visvesvara, 2002).

6.2 Future research

The work generated in this thesis has prompted several questions that align with the three main themes outlined above. In general, the research surrounding the use of *L. salmonae* as a model for environmentally resistant pathogens in an IMTA setting would benefit from observations completed on a fish farm. Additionally, it would be interesting to determine the viability of environmentally resistant pathogens such as *L. salmonae* in other commonly cultured bivalves. The digestive modalities for other filtering bivalves such as clams and oysters can be different, and the pathogens may behave differently when passed through their digestive tracts. Furthermore, it was determined that the majority of spores that were expelled in mussel feces were viable. It can be assumed that viable spores contained within mussel feces would sink to the substrate within the fecal pellet and remain in a concentrated region. In this case, the spores would be available for other cultured organisms that primarily feed on the substrate of overhanging fish net-pens and mussel socks (*e.g.,* American lobsters and sea cucumber species). This set up would be ideal for the mitigation of the accumulating waste products that mussels and fish produce, but could cause future issues for *L. salmonae* and other environmentally resistant pathogens that may be of issue in complex aquaculture systems.

Manipulating the LS-RT model further may also identify risk factors in a farm setting. Specifically, it would be ideal to introduce a co-habitation model. Different exposure scenarios could help clarify the disease interactions between mussels and trout. First, it would be interesting to determine whether mussels bioaccumulate spores when exposed to trout while they are shedding spores. Next, determining the risk of infection when naïve fish are placed in tanks with mussels that have been previously exposed to spores would help determine whether mussels are an effective reservoir for MGDS.

Spore dose and exposure interval could also be manipulated to determine relevant concentrations and exposure risks.

In Chapter 3, spores exposed to mussel digestive tissues did not appear to lose *in vitro* viability, or *in vivo* infectivity. The digestive gland of mussels contain lysozymes that have both defensive and digestive function (Mydlarz et al., 2006). It would seem likely that spores internalized by digestive cells would be deactivated by such lysozymes. It could be that the dose of spores exposed to mussels surpassed the capacity at which they could be internalized and digested. It would be interesting to alter the spore dose to determine whether spores can be digested by mussel digestive enzymes. Immunohistochemistry and/or TEM could also be applied to mussels that have ingested spores to determine whether a portion are in fact being internalized and/or digested.

The antigen responsible for the MAb utilized in Chapter 4 has not yet been identified, so this would be a logical next step. Similarly, it would be interesting to perform immunolocalization of the MAb to determine which part of the spore it binds to. It appears that the spore wall is fluorescing during the IFAT procedures, but it would be noteworthy to determine which part of the spore wall is bound. Lastly, formal cross-reactivity studies using the MAb would help determine whether the MAb is specific to *L. salmonae*, or can be used to identify other microsporidian species. Cross-reactivity studies using human-infecting microsporidia have had varied results. Some species, such as *E. cuniculi* have wide reactivity (Niederkorn & Shadduck, 1980), while others show specificity to only one species (*E. bieneusi*; Accoceberry et al., 1999).

It proved difficult to determine whether certain structures within cells *in vitro* were developing parasites. Further observation of the infected cell cultures by TEM is necessary. The MAb developed for *L. salmonae* appears to be specific to components of

the mature spore wall and thus would not be suitable for immunohistochemistry analysis of early developmental stages. It would be ideal to develop a meront-specific MAb to observe developing stages under light microscopy and TEM. Meront specific MAbs have been developed for human-infecting microsporidians (*e.g., E. cuniculi*), so this technique is possible (Fasshauer et al., 2005). Alternatively, ultrastructural *in situ* hybridization (ISH) could help reveal developmental stages in cell cultures. ISH allows for the localization of target RNA or DNA sequences within cells, and has previously been utilized to detect immature stages of *L. salmonae* in tissues (Sánchez et al., 2000). Post-embedding ISH on existing samples is possible but the method has low specificity, as the full length of the target may not be accessible on the surface of a section (Le Guellec, 1998). However, this could be circumvented if the experimental samples have high infection levels. Additionally, probes for other features that indicate parasite development, such as the presence of host ER surrounding suspect meronts, could prove useful.

It appears that RTgill-W1 cells can support xenoma development by *L. salmonae* (Chapter 5). However, development occurred at low levels. The next step is to optimize this model to yield more development with similar spore exposure numbers (10:1 ratio of spores to cells appears to be the maximum exposure). Experimenting with culture media proportions and additives could help optimize the model. Cuomo and colleagues (2012) theorize that the most important step for microsporidians becoming successful obligate intracellular pathogens is to increase the biosynthetic output of the host. Thus, the development of microsporidia within cells requires an increase in metabolic activity and cells may require more energy sources. Also, it would be wise to compare different incubation temperatures to determine whether this influences growth. Additionally, it

was briefly investigated to see if sporulation could be induced in proximity to live cells, using different chemicals. It would be interesting to expand and replicate the experiment to determine if this mode of cell infection would be more useful in the development of a xenoma-proficient laboratory model. Lastly, other cell types may be more permissive to xenoma development. For example, primary cultures, endothelial-derived cells, rainbow trout monocytes, or a combination of cell types may increase xenoma development.

For the preservation of *L. salmonae* spores within phagocytic AED cells, it would be interesting to complete a TEM study of spore condition after cryopreservation. It appeared that viability was still very low in the cryopreserved spores within cells via the PI exclusion test, but the *in vivo* model showed some promise. Understanding the condition of spores after freezing may help determine if the method is viable.

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